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VIRULENCE IN LEPTOSPIRA. I: REACTIONS OF GUINEA-PIGS TO EXPERIMENTAL INFECTION WITH *LEPTOSPIRA ICTEROHAEMORRHAGIAE*

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IT is well known that pathogenic leptospira lose virulence when grown in culture. There have been no studies of the mechanism of virulence, although Dinger (1932) and Chang (1947) claimed, without confirmation, that they could preserve virulence *in vitro* by various means. The aim of this study was to compare the fate of virulent and avirulent *Leptospira icterohaemorrhagiae* in young guinea-pigs and to investigate some host reactions which might determine the outcome of infection.

MATERIALS AND METHODS

Two strains, " Field " and " Jackson ", of the " complete biotype " of *L. icterohaemorrhagiae* were kindly supplied by Dr. J. C. Broom from the collection of the Leptospira Reference Laboratory.

Virulent spirochaetes.—The " Field " strain was virulent for guinea-pigs. It was maintained at maximum virulence by continual passage through successive guinea-pigs of infected blood or liver obtained at death of the infected animal. The leptospira were cultivated periodically from the blood of moribund guinea-pigs and maintained in culture for use in experiments *in vitro*. The number of " culture passages " referred to in the text is the number of subcultures since isolation from the guinea-pig reservoir. The virulence of organisms obtained directly from the animals was of the order of LD_{50} = one organism.

Avirulent spirochaetes.—The " Jackson " strain has been avirulent for young guinea-pigs for many years. A dose of 10^{10} leptospira produced no ill effects, and lesions could not be found in a 300 g. guinea-pig on histological examination of the organs one week after the intraperitoneal injection of 10^9 leptospira.

Culture

The organisms were cultivated in a modified Korthof medium containing salts, 0·08 per cent " Neopeptone " and 10 per cent rabbit serum. Except where stated they were grown at 30°. Cultures were made isotonic when required by adding 1/10th vol. of 8 per cent NaCl.

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Counting

A direct counting method was used. An " Agla " micrometer syringe half filled with liquid paraffin was mounted pointing downwards at an angle of about 45° on a retort stand. The ferrule of a cut-off No. 19 needle attached to the syringe was surrounded with plasticine. An expendable capillary tube 5–10 mm. long, approx. 2–3 mm. in diameter at the lower end, was fitted over the needle and embedded in the plasticine. With the micrometer, 0·01 ml. of the fluid to be counted was drawn into the capillary tube, expelled on a clean slide and covered carefully with a square $\frac{3}{4}$ in. (1·9 cm.) No. 0 coverslip. The used capillary was then discarded. The spirochaetes were counted directly under dark-ground using a 4 mm. or 8 mm. objective and a 15 × eyepiece containing a graticule ruling, of which all but ten central squares were obscured, and observing the usual conventions regarding organisms lying on a line. Five, ten or a larger convenient number of squares were counted, distributed in the four corners and the centre or otherwise evenly and systematically over the coverslip area. The number of spirochaetes per ml. of fluid was obtained by multiplying the average number of organisms per eyepiece square by the number of squares in the coverslip area, for each objective, and by 100 (the reciprocal of the volume of the drop).

Error of this method.—Results were repeatedly reproducible within 10 per cent in duplicates. Motility of the spirochaetes did not introduce difficulties at the magnifications used, but care was necessary to avoid drying or drifting movements. The method may be adapted to counting small numbers of organisms by searching systematically a large number of fields. Counts down to an order of 10^3 or even 10^2 organisms per ml. may be made. Very densely grown cultures may require dilution to a suitable density before counting.

Counting under aseptic conditions.—It was found best to remove aseptically with a sterile Pasteur pipette small aliquots of fluids required to be kept uncontaminated. The aliquot delivered into a Dreyer tube was then treated as described.

Viable count.—Except where stated, the number of spirochaetes given refers to motile organisms; dead organisms were not counted unless total counts were required. Thus " count " means " viable count ".

Staining

Leptospira were stained in smears with Fontana and Giemsa techniques, and in sections by Dobell's modification of the Levaditi method, which allowed counterstaining of the tissues with haematoxylin and eosin (H.E.) if required.

Serological Methods and Controls

Production and titration of antisera

Rabbits previously shown to be free of antibodies to *L. icterohaemorrhagiae* were injected intravenously thrice weekly for three weeks with 12-day cultures of leptospira. Doses of approx. 10^7 organisms were used the first week, and the dose doubled each week as recommended by van Thiel (1948). The sera were titrated in depressions on porcelain tiles or in Dreyer tubes, using doubling dilutions. Live cultures were used as antigens for agglutination-lysis and formolized cultures for agglutination tests; in each case the antigen suspension contained approx. 10^7 organisms per ml.

The rate of appearance of antibodies in rabbits

One rabbit was injected intravenously with 5×10^8 leptospira in 2·5 or 5 ml. of each of the following 12-day cultures: avirulent live, avirulent formolized, virulent live and virulent formolized. Each rabbit was bled a few drops from an ear vein hourly for the first day and at short intervals on successive days. The sera were titrated for agglutinins and lysins. They were produced in parallel in each animal 50–60 hr. after inoculation in each case. The agglutination and lysis titres of each serum rose to a maximum of 16,000 to 500,000 on the 8th–10th day after inoculation. There was no difference between the four animals in the rate of appearance of the antibodies. In each the titre had fallen to a constant level within 20 days. Thus it appears that a single large dose of leptospira will immunize rabbits as effectively as the elaborate schedule used at first. A second dose of an antigen preparation similar to the first was given 100 days after the first injection. There was no significant difference between the responses to virulent or avirulent leptospira.

Serological relationships of virulent and avirulent leptospira

Rabbit antisera prepared against virulent or avirulent leptospira showed no significant differences in agglutination or agglutination-lysis titres when titrated against either virulent or avirulent cultures. Volumes of 0·25 ml. of antiserum diluted 1/100 were cross-absorbed with concentrated washed suspensions containing 5×10^9 live heterologous-strain organisms in 1·25 ml., giving final antiserum concentrations of 1/600 in 1·5 ml. Agglutinins for both homologous and heterologous strains were completely removed. Absorption of lytic antisera (original titre 6400) with washed formolized leptospira completely absorbed both agglutinins and lysins.

Animals

Guinea-pigs of the stock laboratory strain were of stated weight, usually 100–250 g. They were fed on oats, bran and green-feed. They were apparently uniformly susceptible to infection with virulent *L. icterohaemorrhagiae*. Mice used in some experiments were of the stock laboratory strain. They were almost uniformly resistant to leptospiral infection, although some developed a renal carrier state. Rabbits used for immunization weighed 1·5–2 kg.

Peritoneal exudates containing polymorphonuclear leucocytes were produced by injecting intraperitoneally approx. 7·5 ml./100 g. body weight of sterile saline 15–18 hr. before the experiment, and a further 5 ml. 2 hr. before. Mononuclear exudates were produced by injecting intraperitoneally 2 ml. sterile liquid paraffin into guinea-pigs, or 0·2 ml. into mice, 3 days before the experiment.

Intraportal injection

Under ether anaesthesia an upper right paramedian incision 3 cm. long was made in a guinea-pig. The portal vein was exposed on the dorsal surface of the pancreas and ligated just above the entrance of the splenic vein. About 1 cm. of the end of a cannula of polythene tubing of approx. 1 mm. internal diameter and 6 cm. long, fitted tightly over a No. 19 needle attached to a syringe and filled to the tip with the material to be injected at 37°, was inserted and tied into the portal vein through a nick just proximal to the ligature.

Leptospirosis in guinea-pigs

Typically, guinea-pigs of 150 g. infected intraperitoneally with 10^6 or more virulent leptospira appeared ill on the 3rd–4th day. They became febrile, pale and anorexic. Jaundice usually appeared about the 4th–5th day, preceding death by about 12 hr. and increasing rapidly together with a terminal fall in temperature. The most prominent pathological appearance at autopsy, apart from jaundice, was widespread petechial, sometimes confluent haemorrhages, especially in the lung, abdominal wall muscle and retroperitoneal fat. Renal tubular necrosis was invariably present leading to death from uraemia; nitrogen retention was demonstrable about 24 hr. after infection and rose terminally to a blood urea level of over 200 mg. per 100 ml. Spirochaetes could always be found in blood cultures throughout the infection. At death, the blood, liver and areas of haemorrhage were swarming with spirochaetes which were easily seen in smaller numbers throughout all tissues examined.

EXPERIMENTS

The Fate of Leptospira Injected Intraperitoneally

Two groups each of six guinea-pigs weighing 250 g. were injected intraperitoneally with 2×10^7 leptospira from a 5-day culture. One group received virulent organisms (6th culture passage), the other avirulent organisms. One animal in each group was killed with ether at intervals of 2, 26, 40, 66, 90 and 96 hr. after injection. Sections stained by Dobell's method and H.E. were prepared from skin, abdominal wall muscle, mesenteric lymph nodes, retroperitoneal fat, kidney, adrenal, liver, stomach wall, bladder, lungs and heart, and examined for the presence of spirochaetes and for lesions.

The results of the experiment using virulent spirochaetes are shown in the Table. Spirochaetes undergoing granular degeneration were commonly seen in

tissue sections taken at all stages of infection (Fig. 1). In the final stages of degeneration typical round black (argyrophil) granules, sometimes seen in phagocytes, represented the remains of the leptospira.

TABLE—*Time of Appearance of Spirochaetes and of Histological Lesions in* 250 *g. Guinea-pigs. Injected I.P. with* 2×10^7 *Virulent* L. icterohaemorrhagiae.

	First appearance (hr. after inoculation) of				
	Spirochaetes in				
	Tissue	Blood vessels	Degenerating spirochaetes	Haemorrhages	Degenerative lesions
Liver	2	2	2	40	40
Kidney	2	2	—	40	26
Lymph nodes	40	40	26	—	—
Retroperitoneal fat	66	26	66	40	—
Lung	90	26	26	26	—
Muscle	90	26	66	66	40
Spleen	90	90	90	—	—

— = not observed.

Avirulent spirochaetes were found in the liver, kidney and lymph node 2 hr. after injection. Twenty-six hours after injection spirochaetes could not be found at all, but degenerating forms and granules were found in the lymph node. Avirulent organisms caused no apparent histological changes. Neither virulent nor avirulent organisms produced any significant local inflammatory response. Although there was undoubtedly some phagocytosis in the lymph nodes and in Kupffer cells in the liver, virulent leptospira apparently grew to great numbers in the liver. They could be found throughout the infection in blood vessels in sections. Organs other than the liver, kidney, spleen and lymph nodes appeared to be invaded by spirochaetes carried into tissue spaces by blood extravasated in haemorrhages or in exudates. Leptospira appeared to be phagocytosed either in reticulo-endothelial tissues during massive bacteraemia, as in the terminal phases of infection, or by phagocytes active in areas of haemorrhage, which contained many organisms.

The Fate of Leptospira Injected Intravenously

In an experiment similar to the foregoing, groups of guinea-pigs were injected intravenously. Of a concentrated even saline suspension of leptospira (second culture passage from a 10-day virulent culture), 1 ml. containing 10^9 leptospira was injected intravenously into each of three 250 g. guinea-pigs. A similar injection of avirulent organisms was made into three other guinea-pigs. Degeneration forms were very scarce in these suspensions. One of each group of animals was killed with ether 30 min., 90 min. and 5 hr. after injection. Sections stained by

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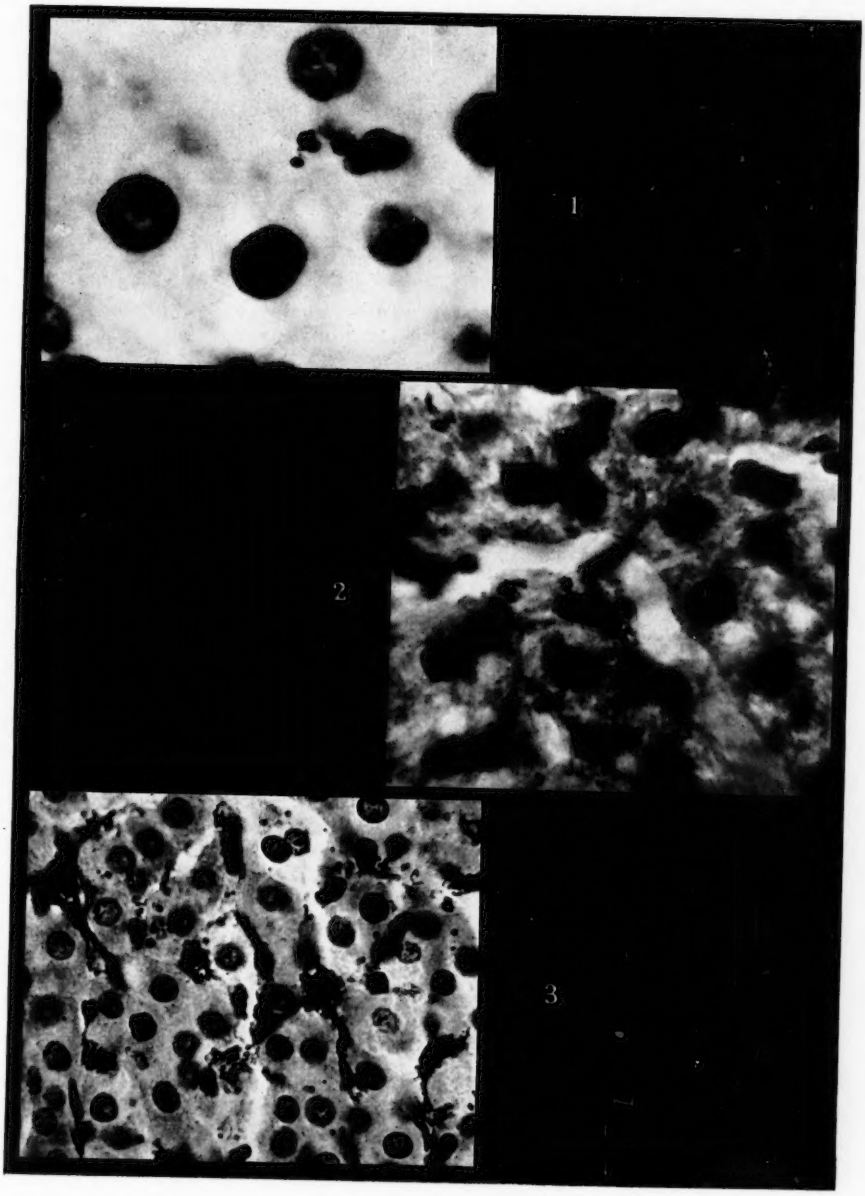
EXPLANATION OF PLATE

FIG. 1.—A degenerating leptospira curling up into a granule in the liver. Dobell and H.E. × 1500.

FIG. 2.—A Kupffer cell in a guinea-pig 30 min. after the intravenous injection of virulent leptospira. Ingested leptospira may be seen beginning to curl up into granules. × 950.

FIG. 3.—Kupffer cells containing ingested leptospira in the liver of a guinea-pig after intraportal injections of washed virulent leptospira followed by 10 ml. saline. × 550.



Faine.

Dobell's method and H.E. were prepared from liver, kidney, spleen, lung and a mesenteric lymph node of each animal, and examined for spirochaetes and for lesions.

The results with virulent and avirulent organisms were almost identical. Thirty minutes after injection intact and degenerating forms of spirochaetes were found mainly interstitially and in Kupffer cells in the liver (Fig. 2) but also sparsely in lung, kidney and spleen. A few degenerating forms were seen phagocytosed in the lymph node. After 90 min. fewer of either form of leptospira were found in the liver. Degenerated forms only were seen in the lymph node, lung and kidney. In the spleen there was a greatly increased number of intact and degenerating organisms, the latter mainly inside phagocytes. After 5 hr. the distribution was still the same, except that the spleen now appeared to be infiltrated with numbers of mononuclear phagocytes containing many black granules representing phagocytosed degenerated spirochaetes.

It is concluded from this experiment that virulent and avirulent leptospira were phagocytosed by Kupffer cells in the liver and by macrophages in other tissues, both before and after degeneration into granular forms, soon after entry into other tissues. The phagocytes, carrying with them ingested spirochaetal débris, migrated to the spleen or lymph node, where they could be found after 5 hr. By this time the concentration of organisms in the liver had decreased. Surviving virulent leptospira remained to establish themselves in the liver. Degenerated leptospira appeared to behave like any other injected foreign particles.

Phagocytosis of Leptospira

Phagocytosis by Kupffer cells

Of concentrated saline suspensions of virulent 8-day cultures (2nd and 3rd culture passage) or of avirulent cultures, 2–5 ml. containing approx. 10^9 leptospira were injected over 10 min. into the portal vein of 200-g. guinea-pigs. The results with virulent or avirulent organisms were similar. Sections of liver excised 5–10 min. after injection showed large numbers of spirochaetes in the sinusoids, mainly trapped in Kupffer cells. Sections of spleen and lung showed few spirochaetes and no spirochaetal débris. In a similar experiment the injection of spirochaetes was followed by an injection over 10 min. of 10 ml. of warm saline through the same cannula. Leptospira were found attached to and apparently inside Kupffer cells in sections of liver excised at the end of the experiment.

Phagocytosis by leucocytes

Negative results were obtained in many experiments *in vivo* and *in vitro* where phagocytic leucocytes were mixed with leptospira under conditions designed to promote phagocytosis. Stavitsky (1948) has reviewed conflicting evidence concerning phagocytosis of leptospira. In view of the lack of confirmation of the only positive observations of phagocytosis of leptospira by leucocytes (Corrales, 1919 ; Martin and Pettit, 1919) the details are of interest.

Experiments in vivo.—Negative results were obtained in two series of experiments : (*a*) Guinea-pigs injected intraperitoneally with saline 18 hr. beforehand were injected intraperitoneally with 8–12 day cultures of virulent (2nd, 3rd and 6th culture passage) or avirulent leptospira. Samples of the peritoneal exudate, rich in polymorphs, were removed at intervals from 15 min. to $4\frac{1}{4}$ hr. after

infection and examined (*i*) directly under dark-ground in sealed preparations at 37°, and (*ii*) by Fontana or Giemsa staining of smears. Intraperitoneal injection of 0·1 ml. or 1·0 ml. of immune rabbit serum (agglutinin titre 5000) caused lysis of the spirochaetes without inducing phagocytosis. (*b*) Similar experiments done on guinea-pigs with a mononuclear peritoneal exudate 3 days old.

Experiments in vitro.—In two series of experiments, 0·1 ml. of 10-day isotonic (*a*) live or (*b*) heat-killed cultures were each incubated at 37° with 1 ml. vol. each of whole polymorphonuclear peritoneal exudate from guinea-pigs, a saline suspension of cells from the exudate, and the supernatant, to give final concentrations of leptospira in two groups of (*i*) $3{\cdot}5 \times 10^6$ per ml. and (*ii*) 2×10^3 per ml. In no case was there a significant reduction of numbers after standing, rotating or centrifuging, for up to 1 hr. Negative results were also obtained in similar experiments using mouse peritoneal exudates. Phagocytosis was never observed in the following preparations: (*a*) that of Nelson (1953) for demonstrating immune adherence, using either human or guinea-pig blood; (*b*) peritoneal exudates to which 10^8 leptospira per ml. had been added, the exudate being allowed to clot between a slide and coverslip and observed under dark-ground at 37°; (*c*) preparations on a thin slide spread with a thin film of 50 per cent serum agar on which a small drop of peritoneal exudate and spirochaetes was placed and covered with a No. 0 coverslip; using a 4 mm. objective, flattened amoeboid polymorphs and motile spirochaetes could be observed in contact, in one plane of focus; (*d*) fibrin mesh preparations for observing chemotaxis (Harris, 1953), in which a small drop of centrifuged leptospira was deposited in the centre of the preparation; chemotaxis was not seen.

In all the above preparations active phagocytes avidly engulfed any other bacteria added. The addition of neither immune serum, which caused eventual lysis of the leptospira, nor crystalline lysozyme to a final concentration of 1/1000 (Gladstone and Johnston, 1955) induced phagocytosis.

No tests were done *in vitro* with mononuclear peritoneal exudates.

DISCUSSION

Standard methods demonstrated no serological differences between virulent and avirulent leptospira which might determine virulence. More elaborate methods appear to be less sensitive than those of agglutination and agglutination-lysis (Schneider, 1955; Chang and McComb, 1954). There appeared to be no difference between the abilities of virulent and avirulent organisms either to penetrate from the peritoneal cavity after intraperitoneal injection or to be engulfed by Küpffer cells in the liver. The sole significant difference appears to be in the ability to survive in the infected animal.

Phagocytosis of leptospira was not observed directly in numerous studies by Stavitsky (1945) although Corrales (1919) and Martin and Pettit (1919) reported its occurrence. These reports could not be confirmed in numerous experiments. However, degeneration of leptospira in tissues into granules has been known since the earliest observations (Kaneko and Okuda, 1917). Zuelzer (1918) and Basile (1921) stated that these granules could be found in phagocytes in tissue sections. These results have been confirmed here, but phagocytosis of leptospira by leucocytes could not be seen either *in vivo* or *in vitro* under a variety of conditions favourable for phagocytosis of other bacteria. Apparently fixed phagocytes

such as Kupffer cells are able to engulf leptospira, at least under the experimental conditions provided, and probably also whenever there is a high local concentration of circulating spirochaetes such as is seen in the terminal phases of infection.

It is significant that chemotaxis could not be demonstrated, using a reliable technique, especially as there is apparently neither phagocytosis nor an inflammatory response to the presence of the spirochaetes. Such leucocytic infiltration as may be seen occasionally in the liver or kidneys may well be a result of the products of tissue breakdown as it does not occur early in the infection even in the presence of large numbers of organisms.

SUMMARY

The fate of virulent and avirulent *Leptospira icterohaemorrhagiae* injected into young guinea-pigs was followed histologically. Both strains behaved in the same way except that virulent organisms survived while avirulent organisms did not.

Using suitable techniques, phagocytosis of or chemotaxis by virulent or avirulent organisms was not seen *in vitro*. Both strains were taken up *in vivo* by fixed phagocytes in reticulo-endothelial tissues, but apparently not by leucocytes. Virulent and non-virulent organisms appeared to be serologically identical.

It is a pleasure to acknowledge thanks to Dr. G. P. Gladstone for his valued advice and criticism, and to Mr. H. Axtell and Mrs. E. Hanley for technical assistance.

Fig. 1 was photographed by Mr. B. H. Glass, Fig. 2 and 3 by Mrs. Daphne Lemon.

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VIRULENCE IN LEPTOSPIRA. II: THE GROWTH *IN VIVO* OF VIRULENT *LEPTOSPIRA ICTEROHAEMORRHAGIAE*

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IN a previous paper (Faine, 1957) it was shown that in contrast with avirulent organisms, virulent leptospira survived and multiplied in susceptible animals. This paper describes quantitative studies of the growth of virulent *Leptospira icterohaemorrhagiae* in young guinea-pigs.

METHODS AND MATERIALS

The strains of leptospira, culture media, counting methods and animals were as described in the foregoing paper.

Counting of leptospira in guinea-pigs

Young guinea-pigs infected intraperitoneally with virulent leptospira were exsanguinated at the required times and autopsied. Histological studies had shown that all but a negligible proportion of the leptospira would be found in the blood, liver, kidneys, adrenals, spleen, lungs and heart. These organs were therefore removed with any exudates, placed in a mortar 15 cm. in diameter and ground with sand into a smooth paste. Sterile isotonic saline was added gradually until the fluid tissue suspension could be transferred to a measuring cylinder. The mortar and pestle were washed three times with saline and the washings added to the suspension in the measuring cylinder to make an accurately noted total volume of 60–100 ml. An aliquot of the thoroughly mixed suspension was removed, suitably diluted, the leptospira counted and the numbers present in the whole suspension estimated. This number or "count" was taken as a close approximation to the total numbers of organisms in the whole animal. The lower limit of the counting method was of the order of 0·5–1 million leptospira per animal. Counts of individual organs were made in the same way by grinding them into a suspension, diluting and counting.

The error of the method is of an order of twofold, but this is small compared with the differences between the numbers of organisms in the samples. Duplicate counts on pairs of animals gave close agreement (Fig. 1) at high and low values. Recovery experiments were unsatisfactory because of the immediate fall in the numbers of organisms after injection (Table I).

EXPERIMENTS

The Growth of Virulent Leptospira in vivo

Growth curves in vivo

The growth of leptospira *in vivo* was followed quantitatively by injecting intraperitoneally known numbers of organisms into groups of guinea-pigs and counting the total number of organisms in pairs of animals killed at known intervals after infection, as described under Methods.

Intraperitoneal injection of large numbers of leptospira ($2–8 \times 10^7$) of the animal passage strain always resulted in an immediate fall in numbers, probably

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due to phagocytosis in the liver. This was followed in about 24 hr. by a logarithmic increase resulting in death after approx. 50 hr. In a series of experiments doses of 2×10^3, 10^6, 2×10^6, 5×10^7 and $8{\cdot}5 \times 10^7$ leptospira of

TABLE I.—*The Fall in Numbers of Leptospira in Guinea-pigs Weighing* 200–250 *g. after I.P. Injection of Virulent Organisms of the Strain Maintained by Animal Passage.*

Experiment	Millions of leptospira injected	Hr. after injection	Millions of leptospira recovered
A	20	2	1·25 ; 3·5
A	20	4	0·65 ; 0·8
B	80	12	0·9
C	65	24	0·5

Leptospira counted in a suspension of liver, kidneys, adrenals, heart, lungs, blood and peritoneal fluid, except in experiment A. In experiment A the 6–7 ml. of peritoneal fluid, which was counted separately, contained all the organisms ; the other organs contained less than 2×10^6. Leptospira were never found in bladder urine.

the strain maintained in guinea-pigs were injected intraperitoneally into groups of young guinea-pigs. Pairs of animals were killed at intervals and the number of spirochaetes in each animal counted. The counts in each group at intervals are plotted in Fig. 1 as *in vivo* growth curves, showing that growth was logarithmic. The growth curves were fitted to the observed points as regression lines calculated by the method of least squares and correspond to generation times of 11·6, 8·7, 5·8 and 7 (average 8·3) hr. for inoculations of 2×10^3, 10^6, 2×10^6 and $8{\cdot}5 \times 10^7$ leptospira, respectively. A similar experiment was carried out injecting doses from 10–10^8 leptospira from an 11-day culture in its 9th culture passage, with an LD_{50} of approx. 10 organisms. In comparison with the previous experiment the growth curves were similar in slope but delayed approx. 24 hr.

Relationship between lesions and the numbers of spirochaetes

Fig. 1 showed that animals inoculated with smaller doses took longer to become ill and die, but contained at or near death numbers of leptospira comparable with those of animals injected with larger doses. The first appearance of haemorrhages in all the animals coincided with counts of approx. 1×10^6. Higher counts later in the infection were associated with more severe lesions and haemorrhages, the extent of which were assessed subjectively without knowledge of what the counts would be.

Distribution of leptospira in the tissues

The tissue distribution of the leptospira differed according to the stage of the infection. In typically severely jaundiced animals killed in the defervescence stage, perhaps 5 hr. before death would otherwise have occurred, 50–70 per cent of the leptospira were circulating in the blood. The liver contained $20{\cdot}4 \times 10^6$ or 82 per cent of the leptospira in the tissues while the adrenal contained $1{\cdot}5 \times 10^6$ (6 per cent) and the kidney $1{\cdot}2 \times 10^6$ (5 per cent) of a total of approx. 25×10^6. These figures agree with the subjective estimate of the tissue distribution of leptospira made in histological studies (Faine, 1957).

The Survival Time of Guinea-pigs Infected with Virulent Leptospira

Fig. 1 showed that guinea-pigs infected intraperitoneally with leptospira of given virulence died after an interval which varied with the dose. Studies were made of the relationship between dosage and survival time, measured as the 50 per cent survival time (ST), the time from infection until the death of 50 per cent (calculated) of inoculated animals. Deaths in each dose-group usually occurred within 24-36 hr. of the ST. The graph of ST plotted against the logarithm of the dose is referred to as the ST : D curve.

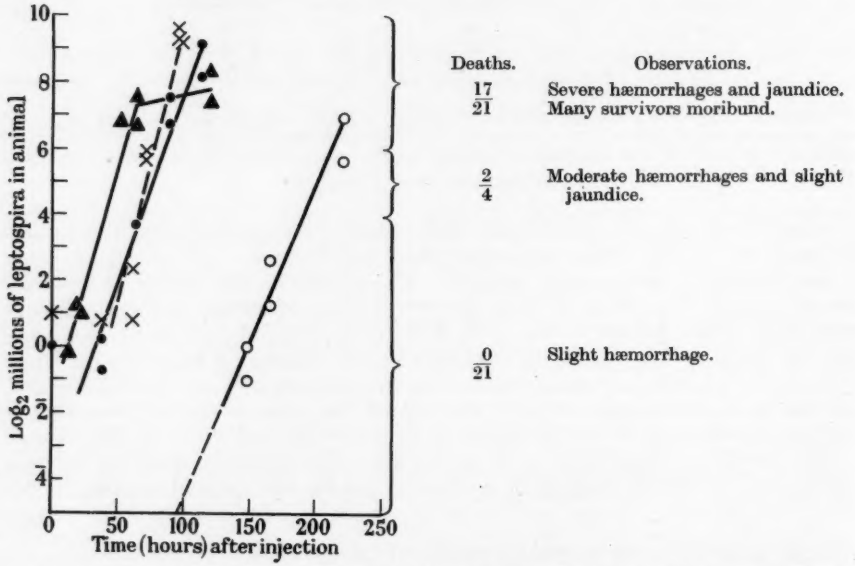

FIG. 1.—The growth curves *in vivo* of *Leptospira icterohaemorrhagiae*. Strain maintained by animal passage and injected I.P. in guinea-pigs weighing 200–250 g.

○——○ Injected with 2×10^3 leptospira. Generation time 11·6 hr.
●——● Injected with 10^6 leptospira. Generation time 8·7 hr.
×——× Injected with 2×10^6 leptospira. Generation time 5·8 hr.
▲——▲ Injected with $5–8·5 \times 10^7$ leptospira. Generation time 7·0 hr.

Guinea-pigs were injected intraperitoneally with various culture passages of the Field strain, of various degrees of virulence. The time of death of the guinea-pigs was noted in several experiments and the data obtained are shown in Fig. 2. In experiment B where agglutinin titres were also measured, only 2×10^6 or fewer leptospira were found in animals dying later than the 8th day after doses of 10^8 leptospira or less, while the antibody titres were 1/100.

In similar experiments a strain subcultivated for $2\frac{1}{2}$ years was found to have an LD_{50} for 180 g. guinea-pigs of $> 10^9$ leptospira. The only animal which died did so on the 11th day after 10^7 leptospira had been injected. The count was fewer than 2×10^6 leptospira, and the agglutinin titre was > 100. The

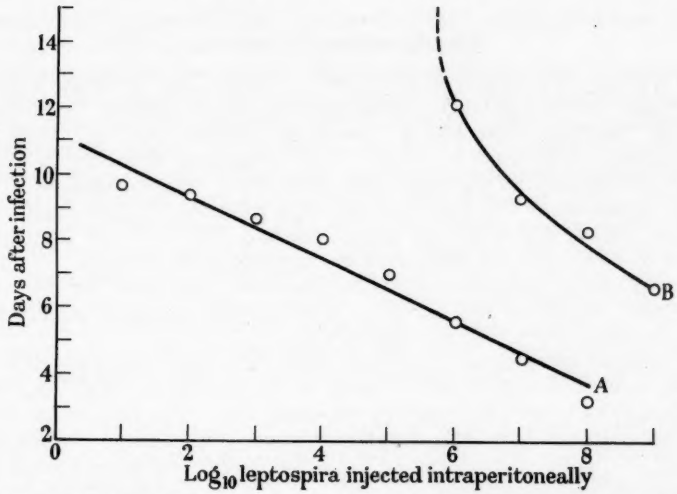

FIG. 2.—The relationship between 50 per cent survival-time and log dose in guinea-pigs inoculated with virulent *L. icterohaemorrhagiae*.

A. Average of data from strain maintained by guinea-pig passage (LD_{50} approx. 2 organisms), and strains of LD_{50} approx. 10 and 100 organisms. 100–250 g. guinea-pigs. Slope $-0{\cdot}95 \pm 0{\cdot}054$, intercept $11{\cdot}1 \pm 0{\cdot}7$. Results from 11–54 animals per dose-group. Total 195 animals.

B. Strain in 18th culture passage; $LD_{50} = 4 \times 10^5$ organisms. 180 g. guinea-pigs. Results from 3–6 animals per dose-group. Total 29 animals.

agglutinin titres from experiment B and other experiments are given in Table II which shows that antibody appeared more rapidly with an inoculum of $> 10^5$ organisms than with one of 10^4 or less.

TABLE II.—*The Rate of Appearance of Antibody in the Serum of Guinea-pigs Weighing* 150–250 *g. after I.P. Injection of Live Virulent* L. icterohaemorrhagiae.

		Number of animals with agglutinin titre indicated on days after infection								
		8		9		10	12		13	20
	Dose inoculated*:	A	B	A	B	A	A	B	A	A
Reciprocal of titre	0	16	—	7	—	3	4	—	1	—
	20 or less	—	—	7	—	—	—	—	2	—
	32	—	—	—	—	—	1	—	1	—
	40	—	—	3	3	—	—	—	—	—
	80	—	—	2	3	—	—	6	—	—
	100	—	3	—	—	—	—	—	—	—
	128	—	—	—	—	1	3	6	—	6
	200 or more	—	—	2	1	—	—	6	7	—
	Total number of animals	16	3	21	6	4	8	18	11	6

* A = 10^4 or less; B = 10^5 or more.

The Regaining of Virulence

The virulence of a culture of leptospira of known low virulence was re-tested after a single passage through young guinea-pigs as follows. Groups of four guinea-pigs weighing 180–220 g. (average approx. 200 g.) were injected intraperitoneally with from 5×10^8–5×10^1 organisms from an 11-day culture of a strain cultivated for $1\frac{1}{2}$ years and in its 19th culture passage. The LD_{50} was $> 10^8$ organisms. On the 10th day after infection the liver and kidneys of a moribund guinea-pig from the group injected with 5×10^7 leptospira were suspended in saline and the leptospira in the suspension counted. No leptospira could be seen after a prolonged search, so that fewer than 2500 per ml. could have been present. Serial ten-fold dilutions were injected intraperitoneally into each of a group of guinea-pigs in 1 ml. amounts.

Many guinea-pigs died of typical leptospirosis on the 10th–14th day. Out of 6 animals, 4, 6, 3, 2 and 2 died in each of the groups injected with undiluted suspension and 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} respectively. The LD_{50} of the suspension was a dilution of 1/280, corresponding to fewer than 9 organisms. This is very close to the LD_{50} of approx. 2 organisms for the strain maintained at maximum virulence by continual guinea-pig passage.

DISCUSSION

Fig. 1 indicated a relationship between the numbers of organisms, lesions and death. This suggests that a certain minimum number of spirochaetes, about a million, are necessary to produce lesions, which cause death after a lag of up to about three days. During this time the full pathological-physiological effects develop. Death occurred about a threshold concentration of an order of 10^8 leptospira per animal. Analysis of Fig. 1 and 2 shows that the time taken to reach this concentration for a given dose can be calculated from the growth rate *in vivo* and agrees approximately with the time expected from the ST.

Curve A in Fig. 2 shows that the ST : D for three strains of highly virulent leptospira (LD_{50} 2, 10 and 100 organisms) has a slope of $-0{\cdot}95 \pm 0{\cdot}054$. Fig. 1 also shows that the growth curves *in vivo* ran parallel following inoculation with various doses. The total growth achieved *in vivo* was of the same order in all animals, regardless of the dose inoculated. The growth curve of a strain with an LD_{50} of approx. 10 was parallel with but about 24 hours later than that of the strain maintained in animals. From these facts it is possible to deduce that there is a ten-fold increase of leptospira *in vivo* in 0·95 days, corresponding to a generation time of approx. 7 hours, which is close to the average of 8·3 hours found. Similar deductions of the growth rate of tubercle bacilli in mice have been made from the same relationship between survival time and log. dose by Youmans and Youmans (1951).

An explanation for the curved ST : D in experiment B in Fig. 2 is that the LD_{50} is relatively high, so that a very small proportion of the large numbers of organisms injected is virulent. The course of the disease is therefore delayed as the number of virulent organisms has not reached levels associated with fatal lesions before antibodies appear and modify the outcome of the infection. This factor did not influence the ST : D of experiment A because such a large proportion of the spirochaetes injected was virulent that even with small doses most of the

animals were dead by the 9th day. Antibodies were not found before that time when small doses of spirochaetes were injected (Table II).

Death occurred in the absence of a large number of spirochaetes and three days after antibody first appeared in animals injected with doses near the LD_{50}. This is not inconsistent with the pathology of leptospirosis. The lesions may progress past an irreversible point. For example, in guinea-pigs infected with approx. 10^6 virulent (animal passage strain) leptospira the organisms may be eliminated by treatment with erythromycin, but unless this is begun within the first 72 hours the course of the disease is not altered and death ensues about 100 hours after inoculation (Faine and Kaipainen, 1955). Inada, Ido, Hoki, Kaneko and Ito (1916) found that serum therapy was ineffective in guinea-pigs once jaundice appeared, about 24 hours before death. Also, in fatal Weil's disease in man spirochaetes are seldom found at autopsy when antibody is present. Ido, Hoki, Ito and Wani (1916) and Kaneko and Okuda (1917) showed that spirochaetes disappeared from the tissues about the 8th day, coincident with the appearance of antibody.

The disease in guinea-pigs inoculated with a large dose of leptospira of low virulence therefore resembles more closely Weil's disease in man than the disease produced in guinea-pigs by small or large doses of highly virulent *L. icterohaemorrhagiae*. It is suggested that, in infection with highly virulent leptospira, survival after sub-lethal doses is due to the absence of virulent leptospira from the inoculum. Survival in infection with relatively less virulent cultures is partly a result of the intervention of antibody. A greater variation in measurements of virulence by LD_{50} may be anticipated in the latter case, where the appearance of antibody may play a part in determining survival, than in the former. The failure reported by some authors to restore virulence by animal passage could thus be explained by assuming that the culture did not contain virulent organisms in the dose given. The need to use large doses and several animals in attempting to make avirulent cultures virulent by animal passage is obvious, but these measures will not ensure success.

It is possible to erect a hypothesis that virulence is a result of the selective multiplication *in vivo* of virulent leptospira. Confirmatory evidence is provided by the fact that maximum virulence may be regained after a single animal passage. Presumably virulence is lost in culture by a postulated mutation to avirulent forms. In this connection it is of interest that, in unpublished experiments, avirulent leptospira of the " Jackson " strain fully adapted to cultural conditions grew with a generation time of 7–8 hours *in vitro* comparable with that of virulent leptospira *in vivo* of 8·3 hours. The shortest generation time recorded for freshly-isolated virulent leptospira grown in the same medium at 37° was 17–21 hours. This suggests that virulent leptospira which multiply *in vivo* are " adapted " to grow in the host, in contrast to avirulent leptospira which are " adapted " to grow in culture.

SUMMARY

The growth of virulent *Leptospira icterohaemorrhagiae* in young guinea-pigs was studied by counting the organisms in the animals at intervals after intraperitoneal injection. There was an initial fall in numbers followed by a logarithmic increase representing an average generation time *in vivo* of 8·3 hours.

The severity of the lesions appeared to increase with the numbers of organisms present until death occurred with 10^8–10^9 leptospira per animal.

There was a constant relationship between survival time after intraperitoneal injection and the logarithm of the dose, for strains of given virulence. This relationship was correlated with the growth rate *in vivo*.

The experiments suggest that virulence is the result of differential survival *in vivo* of virulent organisms approximately one of which is contained in the number represented by the LD_{50}.

The infection of guinea-pigs with relatively avirulent leptospira results in conditions similar to Weil's disease in man, in contrast to the acute illness seen after infection with highly virulent organisms.

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STUDIES ON INTRACEREBRAL TYPHOID INFECTION IN MICE. I: CHARACTERISTICS OF THE INFECTION

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THE experimental infection of mice with *Salmonella typhi* has now been used for over two decades, principally in the study of the interaction of typhoid antigens and antibodies, and in the assay of the protective potency of typhoid vaccines. This infection is produced by intraperitoneal inoculation of mice with bacilli (Grinnell, 1932) suspended in either saline or mucin. Even the most virulent strains of *Salm. typhi* fail to infect fatally unless large numbers, of the order of 70 million or more, are employed. Indeed, some workers have routinely used a challenge dose of approximately 250 million bacilli (Felix and Pitt, 1935; Felix and Anderson, 1951). However, if the organisms are inoculated as a suspension in gastric mucin, a substance which depresses host defences, relatively few organisms, 1000 or less, suffice to produce a fatal infection (Rake, 1935). Relatively little attention has been given to the nature of the infection initiated by either type of challenge. However, it was observed early that symptoms of acute intoxication arose shortly after the injection of a lethal dose of typhoid bacilli and that nearly all animals succumbed within 24–36 hr. Survivors became asymptomatic rapidly and such animals were found to harbour appreciable numbers of organisms (Ørskov and Kauffmann, 1936). Certain aspects of the infection produced intraperitoneally, such as the overwhelmingly toxic death and either the magnitude of the challenge dose required or the employment of a host-resistance depressant such as mucin, have resulted in criticism of conclusions based on the use of this technique.

Another method, that of infecting mice with *Salm. typhi* by intracerebral injection, was first described by Norton and Dingle (1935). This showed promise in that it was possible to infect mice with a moderate number of bacilli without the use of mucin. The intracerebral route has since proved to be the one of choice for the study of infection and immunity to *Haemophilus pertussis*, and is now the basis for official potency tests of pertussis prophylactic agents (Minimum Requirements: Pertussis Vaccine, 1952). On the other hand, workers concerned with laboratory studies of typhoid infection have generally ignored the intracerebral route of inoculation. We re-investigated this as a possible means of eliminating the features of the intraperitoneal procedure which have been considered most objectionable. It was found that with the use of an appropriate mouse strain and challenge culture, a fatal infection could be established with a small inoculum. This report identifies the factors required for the establishment of this infection and describes certain of its characteristics.

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MATERIALS AND METHODS

Strains of Salm. typhi.—The widely employed vaccine strains, Ty2 and 58, were selected as representing strains containing both Vi and O antigens. Also selected were the international strain O–901 as a pure O strain, and the strain Vi I, as a serologically rough Vi culture containing little or no O antigen. Lastly, a streptomycin-dependent variant was employed which had been isolated from the parent Ty2 strain by our colleague, Dr. L. Baron. This culture was maintained on meat extract agar containing 20 μg. streptomycin per ml.

Preparation and administration of challenge.—Challenge suspensions were prepared by growing cultures on veal infusion agar for 12–15 hr. at 37°. The organisms were harvested and the numbers in the suspensions were standardized spectrophotometrically. Dilutions were made in 1 per cent pancreatic digest of casein (Difco) in saline pH 7·1, so that the desired number of viable bacilli for intracerebral inoculation was contained in 0·03 ml. The number of organisms in the challenge suspensions was checked by plating appropriate dilutions. Challenge suspensions were injected within 1 hr. of preparation. The injections were made intracerebrally through the foramen magnum of mice lightly anaesthetized with ether with short-bevel, ½ inch (0·6 cm.) 25 gauge hypodermic needles and 0·25 ml. tuberculin syringes.

Bacterial counts.—The number of bacilli in the brain and other organs of infected mice were determined as follows: The organ was excised aseptically, ground with sterile sea-sand to a homogeneous paste, and suspended in trypticase-soy broth (Baltimore Biological Laboratories). Serial dilutions of the suspension were made in this broth and in addition appropriate samples were plated on veal infusion agar. The number of organisms in the whole organ was calculated from the plate counts and from the highest dilution of broth exhibiting growth.

Mouse strains.—Animals of both sexes, 5–7 weeks of age and weighing 12–14 g. were used. Eight genetically classified inbred strains of mice were kindly supplied by Dr. George Jay of the National Institutes of Health, Bethesda, Maryland. These are designated in Table I according to the standardized nomenclature for inbred strains of mice (Carter, Dunn, Falconer, Grüneberg, Heston and Snell, 1952). Two additional strains obtained from that organization were randomly-bred Swiss albino mice (GP), and brother-sister matings of the GP mice, referred to as the NIH strain. Six inbred strains were provided by the Department of Animal Husbandry, Walter Reed Army Institute of Research. The history of these strains is recorded at this institution. CFW mice were purchased from Carworth Farms Inc., New York City, New York.

Morbid anatomical examinations.—Those mice destined for histopathological examination were killed with chloroform, autopsied, the brain was removed intact, fixed in neutral formalin, cut coronally into multiple blocks approximately 4 mm. in width, then embedded in paraffin, cut at approximately 7μ and stained with haematoxylin and eosin.

RESULTS

Susceptibility of Different Mouse Strains

The strain of mice generally employed for typhoid studies by one of us (M. L.) has been the well known Bagg strain. Preliminary tests showed that it was possible to produce a fatal infection in these animals by intracerebral inoculation. However, with any of a number of virulent strains of *Salm. typhi*, mortality seldom exceeded 40–50 per cent even when large inocula were used. Consequently, attention was then directed towards the possible importance of genetic differences in mice in determining susceptibility to intracerebral inoculation. A total of 17 strains of mice, most of which were genetically classified, were available for this purpose. In any given test, not less than 20 mice per group were employed. Each mouse strain was tested at least twice and most strains were tested three to five times. It was not possible to carry out comparisons of all strains in a single experiment, but similar test conditions were maintained throughout and

each experiment incorporated the maximum number of strains. The results of this comparison are given in Table I, and show important differences in susceptibility to intracerebral inoculation of approximately 50,000 *Salm. typhi* Ty2, mortality varying from 18 to essentially 100 per cent.

TABLE I.—*Susceptibility of Different Strains of Mice to Intracerebral Typhoid Infection.*

Susceptibility	Strain*	Number of mice tested	Mortality (per cent)
High	BALB/c[1]	227	99·6
	A/He[1]	53	93
	A/L[1]	102	97
	C3H[1]	79	91
	RI[1,2]	75	91
Moderate	DBA[1]	102	82
	CAF1[1]	94	80
	Cinnamon[2]	39	69
	BSVS[2]	39	64
	RN Brown[2]	63	64
	C57BL[1]	70	48
	CFW[3]	57	46
	BALB[2]	115	43
Low	RJ White[2]	33	36
	GP[1]	97	34
	SWR[1]	42	26
	NIH[1]	60	18

All mice challenged with 30,000–50,000 *Salm. typhi* Ty2.
* Obtained from : (1) National Institutes of Health, (2) Walter Reed Army Institute of Research, (3) Carworth Farms.

For convenience in presentation, the mouse strains have been arbitrarily grouped as being of high, moderate or low susceptibility. It is appreciated that were it feasible to carry out complete virulence titrations, *i.e.*, an extended range of challenge doses for each mouse strain, this order and degree of susceptibility probably would shift somewhat. However, it is probable that as the challenge level is reduced, variation in individual mouse susceptibility would become more apparent. Indeed, experiments with BALB/c mice, the strain of highest susceptibility, have shown that while a challenge as small as 20 organisms may induce a fatal infection, the percentage of animals which succumb in any given experiment employing 10–200 cells is highly variable. The variability in distribution of organisms at such low challenge dosages and the heterogeneity of the mouse population probably account for the unpredictability of these results. During the course of this and the accompanying investigation (Landy, Gaines and Sprinz, 1957), 1219 of a total of 1240 normal BALB/c mice inoculated intracerebrally with 5,000–50,000 *Salm. typhi* died, showing that this technique consistently produced a fatal infection. In any case, the results given in Table I serve to emphasize the important part played by genetic constitution in the establishment of a fatal intracerebral infection in the mouse. On the basis of these findings, strain BALB/c was selected for all subsequent work.*

* It is noteworthy that the strain of highest susceptibility (BALB/c) is an inbred variant of the Bagg strain (BALB) used in these laboratories and initially found to be of relatively low susceptibility to intracerebral challenge (43 per cent mortality).

Virulence of Different Strains of Salm. typhi

The availability of a mouse strain of uniformly high susceptibility permitted a direct comparison of the virulence of different strains of *Salm. typhi*. Table II shows the results of one of several experiments in which 4 strains, which have been studied extensively in many laboratories during the past 20 years, and which represent combinations of the major antigenic components of this organism, were compared. It is apparent that strains such as 58 and Ty2 which possess both Vi and O antigens, were highly lethal, since the LD_{50} of both was less than 200 cells. On the other hand, strains containing only one of these antigens showed limited virulence. Thus, the LD_{50} for the serologically rough Vi strain is in excess of 10 million, while that for the O strain is approximately 5 million cells. It is of interest and probably significant that the relative mouse virulence of these strains by the intracerebral route is, for the most part, similar to that by the intraperitoneal route.

TABLE II.—*Virulence of* S. typhi *for BALB/c Mice Inoculated Intracerebrally: Comparison of Strains.*

Strain	Antigenic components	Challenge dose 10,000,000	1,000,000	100,000	10,000	LD_{50}
O–901	O	13/20*	8/20	8/20	5/20	5,000,000
Vi I	Vi	2/20	0/20	2/20	1/20	>10,000,000
		200,000	20,000	2,000	200	
Ty2	Vi+O	19/19	20/20	20/20	15/19	<200
58	Vi+O	20/20	20/20	19/22	13/20	<200

* Fractions = Number of deaths/total number mice.

Relationship between Bacterial Multiplication, Tissue Changes and Mortality in Mice

It was felt that a study of the sequence of events following intracerebral inoculation, such as determination of the rate of multiplication of the inoculum, the dissemination of bacilli in organs and the progressive development of characteristic lesions in the brain, might provide a better understanding of the nature of the infective process. Accordingly, a number of experiments with these objectives in mind were performed, of which the following is typical. Three hundred mice were inoculated intracerebrally with 40,000 *Salm. typhi* Ty2, the animals randomly distributed 5 to a jar, and the jars assembled into groups of 100 mice. One group was employed for the purpose of following bacterial numbers and distribution, another for study of the development of brain lesions, and the third for the mortality rate. At 6 and 12-hour intervals, jars of 5 mice from each of the first 2 groups were selected in accordance with a predetermined schedule and the animals sacrificed for bacteriological and histopathological study, while mortality in the third group was recorded.

Table III shows the multiplication of challenge, the dissemination of bacilli in several organs, as determined by viable count, and the number of mice which succumbed at indicated intervals following challenge. It will be noted that at zero time (actually 10 min. post-challenge), essentially all the inoculum was

accounted for. In the brain significant, but not extensive, multiplication of organisms occurred as early as 12 hr. after inoculation. Numbers increased rather rapidly after 18–24 hr., attaining a maximum of several hundred million bacilli, which was maintained until all animals succumbed. In other experiments, maxima of 500–1000 million organisms were encountered. By 12 hr., bacilli were found in the heart's blood, spleen and liver. In contrast to the findings in the brain, much smaller numbers of bacilli were demonstrable in these organs. The viable counts in this and other experiments ranged from 100 to 10,000 bacilli per ml. of blood or entire spleen or liver. Within these limits the numbers varied widely among mice at any given time and from one time period to another, and thus provided no evidence of progressive increase in total count in these organs. It is likely that the presence of bacilli in spleen and liver reflect their presence in the blood stream rather than separate foci of multiplication.

TABLE III.—*Multiplication and Dissemination of Typhoid Bacilli in Mice Following Intracerebral Inoculation.*

Hours post-challenge	Percentage mortality (cumulative)	Bacteriological findings			
		Brain* (whole organ)	Blood† (per ml.)	Spleen† (whole organ)	Liver† (whole organ).
0	—	40,000	0	0	0
6	0	20,000			
12	0	500,000	600	500	6,000
18	2	12,500,000			
24	6	25,000,000	4,000	3,700	400
36	8	35,000,000			
48	10	35,000,000	3,300	4,000	2,800
60	13	80,000,000			
72	20	79,000,000	5,500	500	7,000
84	42	200,000,000			
96	59	400,000,000	2,000	500	200
108	73	300,000,000			
120	85	350,000,000	500	1,900	3,300
132	95	95,000,000			

All mice challenged with 40,000 *Salm. typhi* Ty2.
* Mean values for groups of 5 mice.
† Mean values for groups of 3 mice.

The only extensive multiplication of the inoculum occurred in the area of deposition, the brain. The relationship between the rate of multiplication in this organ and the concomitant mortality is depicted graphically in the Figure.

There was a lag of approximately 3 days between the development of large numbers of bacilli in the brain and progressive rise in mortality. Few deaths occurred during the first 2 to 3 days although most animals appeared ill as evidenced by a ruffled coat, a gummy exudate sealing the eyelids, a characteristic hunched posture and markedly reduced activity. Beginning on the 3rd or 4th day the animals generally exhibited paralysis and thereafter rapidly succumbed, mortality usually reaching 100 per cent by the 6th to the 10th day depending on the dose.

A smaller inoculum generally produced a similar picture with regard to the course of disease and multiplication of organisms, except for a slower rate of development; the bacterial populations ultimately attained in the brain were comparable to those resulting from inoculation with 50,000 cells.

Examination of brains harvested according to the schedule as outlined previously reveals a characteristic sequence of events in the evolution of the inflammatory process. The size of the inoculum apparently only influences the speed with which the changes occur, but not the sequence as such, nor the pattern and distribution of the lesion or the type of cellular exudate. In an experiment in which 5000 organisms were injected, the first changes noted within a few hours were a fresh haemorrhage along the needle track and an early glial-mesenchymal response. At the same time an early meningitis was present, exudate containing polymorphs and mononuclear cells. From the beginning the meningitis was diffuse and became progressively more severe. Already at 24 hr. the spread of

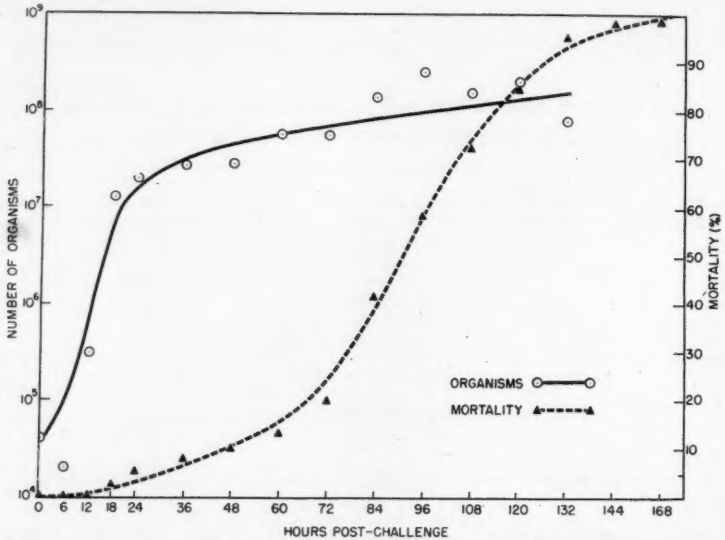
NUMBER OF ORGANISMS
MORTALITY (%)
HOURS POST-CHALLENGE
ORGANISMS
MORTALITY
0 6 12 18 24 36 48 60 72 84 96 108 120 132 144 168
10 20 30 40 50 60 70 80 90

FIGURE.—Bacterial multiplication (in brain) and mortality following intracerebral inoculation of mice with 40,000 *Salm. typhi* Ty2.

the inflammation along the Virchow-Robin spaces was noticeable. At the inoculation site a small abscess had formed. There was a moderate degree of hyperaemia which progressively increased. At 36 hr. this had affected the subependymal vessels and there was a minimal diffuse inflammatory infiltration of the connective tissue stroma of the choroid plexus. The ventricles were still free of exudate. At 48 hr. a significant symmetrical, bilateral extension of the inflammatory process within the ventricular system had taken place, producing an early purulent ependymitis, particularly noticeable in the lateral ventricles. At about 96 hr. the ventricular system was completely filled with pus. There was now a quite extensive liquefaction necrosis of the subependymal brain tissue resulting from an extension of the inflammatory process beyond the confines of the lateral ventricles with separation of the neocortex from the hippocampus. At 120 hr. the intensity of the inflammatory process had further increased. There is evidence of definite obstruction of the ventricular system and distension of the ventricles

with pus. The meningitis had likewise continued to increase in severity. There was some further progression of the inflammatory reaction at the 168-hr. interval, the last studied. The animals at this time were succumbing to a diffuse purulent meningo-encephalitis and particularly to a purulent ependymitis with severe dilatation of the ventricular system due to obstruction. The histological findings correlate well with the clinical course and the mortality curve, as well as with the propagation rate of the organisms.

In as much as endotoxin is recognized as the principal noxious component elaborated by the typhoid bacillus it was of interest to determine the extent to which it contributed to the outcome of the infective process. Observations up to this point had suggested that if endotoxin *per se* played a rôle in the evolution of the process it was only in so far as it was connected with the virulence of the challenge strain which permitted the organism to multiply progressively.

In order to test this assumption experiments were done to ascertain the rôle of endotoxin in the pathogenesis of the intracerebral infection. Purified *Salm. typhi* endotoxin (Landy and Johnson, 1955), in a dosage range of 1 to 30 μg. (estimated to be equal to 0·07 to 2 billion organisms) proved highly toxic but not lethal for mice when injected intracerebrally. The animals showed symptoms indistinguishable from those of infected mice at 2 to 4 days but recovered within a few days. Histological examination gave variable results in two experiments using 15 μg. of endotoxin. In one group of animals a number of deaths resulted from extensive haemorrhage and necrosis of the midbrain, the site into which the injection was made, while in a subsequent experiment using the same dose and the same strain of mice, these findings could not be duplicated. Instead the inflammatory reaction never exceeded the 24-hr. phase of the infection of the brain described above. The peak of the endotoxin response was reached within 24 hr. and subsequently rapid healing took place corresponding to the clinical course. These experiments leave little doubt about the immediate and direct effect of purified endotoxin on brain tissue. However, this effect is rather rapidly dissipated and is not entirely consonant with the slight histological lesion observed. In contrast, in the animals challenged with bacteria, lesions of similar intensity are associated with only a moderate indisposition of the animal.

In other experiments groups of 20 mice each were given 10, 100, or 1000 million heat-killed *Salm. typhi* Ty2 intracerebrally. Relatively little evidence of toxicity was manifested by these animals, and only 2 of the mice receiving the largest dose succumbed. Brains of mice killed 192 hr. following the challenge showed only a slight to moderate hyperaemia. There was a slight haemorrhage at the inoculation site together with a mild glial-mesenchymal response. There was no sign of meningitis or ependymitis. Only those animals receiving the highest dose showed in addition a slight exudate of mononuclear cells in the pia-arachnoid. These findings compare well with the clinical course and show that heat-killed organisms do not elicit a significant inflammatory reaction; they produce less effect than an equivalent amount of purified endotoxin.

Another experiment was designed to determine whether viable cells *per se* are more toxic than those killed by heat. Groups of 40 mice each were inoculated intracerebrally with a streptomycin-dependent variant of the Ty2 strain, *i.e.*, a viable, but non-multiplying challenge. In the groups inoculated with 100 million or less of these organisms, few if any mice died (3 deaths in 40 mice injected with

100 million bacilli). It was only when 1000 million bacilli were administered that a considerable number of deaths (75 per cent) occurred. This experiment was repeated with essentially similar findings.

Histologically the brains of mice inoculated with 100 million of these organisms and killed approximately 7 days later showed fairly massive abscess formation with necrosis of the surrounding brain tissue at the inoculation site in the midbrain. There was a slight to moderate degree of meningitis and ependymitis but no spreading encephalomyelitis. With an inoculum of 1 million bacteria there was, at the same time interval, a distinct tendency towards healing of the needle track with only a slight leucocytic infiltration, while compound granular cells were very much in evidence. There was a sprouting of capillaries and a slight glial-mesenchymal response. This pattern of response differed from the previously described reaction to infection with virulent bacilli in that the inflammation was practically limited to the inoculation site where it was severe with the higher dosage. Massive abscess formation at the inoculation site was only observed in these mice infected with streptomycin-dependent organisms ; it was not seen following inoculation with purified endotoxin. Endotoxin appeared to act primarily on the capillaries and in certain instances caused massive haemorrhage and necrosis. The degree of meningitis and ependymitis seen following inoculation with streptomycin-dependent organisms was distinctly less than that present at the same time interval following inoculation of 5000 *S. typhi* Ty2 as previously described. Endotoxin did not induce ependymitis and only a slight degree of meningitis while the animal inoculated with virulent bacilli succumbed to a spreading, very severe acute purulent meningo-encephalitis with particular involvement of the ventricles. The death of the animals inoculated with viable but non-multiplying organisms was attributable to the massive degeneration of the midbrain, the site of deposition of the inoculum.

DISCUSSION

The evidence presented here shows that under appropriate conditions, mice may be fatally infected with small numbers of *Salm. typhi* inoculated intracerebrally. In addition to a susceptible host, the antigenic composition of the organism is important in producing this fatal infection. The combination of Vi and O antigens in *Salm. typhi* appears to be essential in establishing a fatal infection in the presence of those anti-bacterial factors which are effective against strains containing only Vi or O antigen alone. These observations fully confirm the main facts established for mice infected intraperitoneally, as first reported by Felix and Pitt (1935) more than 20 years ago and subsequently by other workers. It is significant that despite the obvious differences in the nature of the infective processes initiated by these two routes, a fatal infection in both instances is produced only by strains of *Salm. typhi* possessing the two major antigenic components. It is apparent that, regardless of the challenge route, the body defence mechanisms, probably humoral, are capable of controlling infections initiated by Vi or by O strains of *Salm. typhi*. Differences in the virulence of challenge strains may be greatly magnified by the intracerebral route. For example, the LD_{50} of strains Ty2 and O–901, administered intraperitoneally as saline suspensions, differ by approximately 20-fold (Felix and Pitt, 1951), while by the intracerebral route these differences are enlarged to more than 1000-fold (see Table II).

It is obvious that the reaction of the mouse to a given strain of *Salm. typhi* is very different in the two sites. A small inoculum in the peritoneum is rapidly eliminated since the organisms are dispersed throughout the peritoneum and are brought into immediate contact with phagocytic cells. A similar inoculum in the brain of BALB/c mice initially is restricted more or less to the area of deposition. The cellular defence mechanism, the microglia, as well as the blood-borne macrophages and leucccytes are promptly mobilized to cope with the challenge. Nevertheless, the infective process becomes established and progresses to a characteristic meningo-encephalitis. These facts support the view that in the procedure here described the brain does not function merely as a suitable culture medium, but rather that the interaction between host and organism in brain tissue gives rise to a characteristic sequence of events. The primary determinants of the ensuing inflammatory process are the effective multiplication of the micro-organisms and the rate of mobilization of defence mechanisms, cellular and humoral, their interaction and their effect on brain tissue. It is noteworthy that mice inoculated intracerebrally exhibit an early bacteraemia which persists until death. The lesions in the brain are of such magnitude that the bacilli have ready access to the general circulation and it can be safely assumed that the brain serves as a focus from which bacilli continue to enter the blood stream and organs such as spleen and liver. The circulation normally provides an effective clearing mechanism. There is little doubt that this dissemination of organisms is of secondary importance compared to multiplication in the brain, and probably contributes little, if anything, to the fatal result.

The marked variation in susceptibility of genetically different mouse strains is of unusual interest. Genetic differences are known to play an important part in the susceptibility of mice to certain bacterial (Gowen, 1948) and virus (Sabin, 1952) infections. The variation in susceptibility of mice of different genetic constitution as here reported may be a reflection of inherently different levels of natural anti-bacterial factors, differences in the concentration of essential metabolites or variation of susceptibility of brain tissue. At present these remain to be determined.

Results of experiments with isolated endotoxin and viable, non-multiplying, or killed organisms, undertaken to define the rôle of endotoxin in the death of infected animals, indicate that this substance probably is not the major determinant of death. It must nevertheless be remembered that the doses tested correspond only to the numbers of bacilli found at a given time and not necessarily to the total amount of endotoxin formed during fatal infection. Consequently, these experiments do not entirely rule out the possibility of endotoxin being a primary cause of death, since accumulation of endotoxin during the infection undoubtedly results in considerably greater total quantities than were administered. It is not possible to distinguish histologically the leucocytic response produced by endotoxin from that provoked by the organisms themselves.

The important inference to be drawn from the experiments with killed typhoid bacilli, isolated endotoxin, and a streptomycin-dependent variant of this organism is that the lethality of *Salm. typhi* may be attributed mainly to the growth of the organisms in brain tissue. The relatively small initial dose of viable bacilli, which increases in number concomitantly with the progressive fatal infection, in itself attests importance of the changes resulting from this multiplication of organisms in the brain.

SUMMARY

A study was made of the intracerebral infection of mice with *Salm. typhi* in which it was shown that a susceptible strain of mice and a challenge culture possessing both Vi and O antigens were required for the production of an infection which terminated fatally. A great variation in susceptibility of genetically classified mouse strains was found. Under optimal conditions, *i.e.*, use of BALB/c mice and *Salm. typhi* Ty2, few organisms sufficed to initiate a fatal infection.

After intracerebral inoculation with virulent bacilli a period of approximately three days elapsed during which there occurred extensive bacterial multiplication in the brain. This was followed by a progressive rise in mortality, which reached 100 per cent in 6–10 days. The histopathological changes in the brain proceeded at a rate similar to that of bacterial multiplication. The animals finally succumbed to an acute purulent meningo-encephalitis and a secondary cerebral oedema and pyocephalus.

Intracerebral typhoid infection in contrast with intraperitoneal, can be established with small numbers of organisms, requires no adjuvant and produces a progressively developing fatal infection with a well defined incubation period, and characteristic dissemination of organisms and tissue changes.

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STUDIES ON INTRACEREBRAL TYPHOID INFECTION IN MICE. II: IMMUNOLOGICAL FACTORS CONCERNED IN PROTECTION

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In studies with mice infected intraperitoneally, considerable evidence has been accumulated showing that of the two major antigenic components of *Salm. typhi*, the Vi antigen is highly protective (Felix and Pitt, 1934; Longfellow and Luippold, 1943; Landy and Webster, 1952), while the O antigen shows comparatively little immunogenic activity (Landy, Johnson, Webster and Sagin, 1955). Consequently it was important to examine the rôle of these antigenic components in protecting against intracerebral infection, the features and advantages of which are described in the preceding paper (Landy, Gaines and Sprinz, 1957). This report records the results of mouse protection tests with typhoid vaccines, isolated antigens and their corresponding antisera. It is shown that the Vi antigen is the essential component for the production of immunity to intracerebral infection with *Salm. typhi*.

MATERIALS AND METHODS

Isolated antigens.—Purified Vi and O antigens were derived from *Bacterium coli* 5396/38 and from *Salm. typhi* O-901, respectively. The isolation procedures and the chemical and immunological properties of these antigens are described in publications from these laboratories (Webster, Landy and Freeman, 1952; Webster, Sagin, Landy and Johnson, 1955; Landy and Webster, 1952; Landy *et al.* 1955).

Bacterial vaccines.—Heat-killed, phenol-preserved typhoid vaccine, acetone-dehydrated typhoid vaccine, and dehydrated V-form *Bact. coli* and *Bact. ballerup* were employed. The preparation and immunogenic properties of these agents have been reported (Landy, 1952, 1953). Commercial T.A.B. vaccine (Lilly & Co.) was purchased on the open market.

Mouse protection tests.—Details of the manner in which active and passive immunity tests were performed in mice have been reported previously (Landy, 1953). In the present work these procedures generally were adhered to, with the following exceptions: (*a*) Mice of the BALB/c strain, weighing 12–14 g. and of both sexes, purchased from the Jackson Memorial Laboratories, Bar Harbor, Maine, or donated by the National Institutes of Health, Bethesda, Maryland,† were employed throughout. (*b*) Unless otherwise noted, vaccines or isolated antigens were injected intraperitoneally 6 days prior to challenge, while sera were administered intraperitoneally 1 hr. before challenge. (*c*) The preparation and administration of challenge suspensions of *Salm. typhi* Ty2 are described in the preceding paper.

Bacteriological and histopathological examinations.—The procedures employed in the examination of animal tissues are given in the preceding paper.

RESULTS

Protection by typhoid vaccine as a function of Vi antigen content.—In previous studies on the protective potency of various typhoid vaccines against intraperitoneal inoculation, evidence was obtained that protection was related to the

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† We are indebted to Dr. George Jay for the supply of these animals.

amount of Vi antigen in the vaccines (Landy, 1953). Corresponding tests were conducted in mice actively immunized with these vaccines, of varied Vi antigen content, and challenged intracerebrally. The results of these tests are presented in Table I. It is apparent that for all practical purposes the O

TABLE I.—*Immunogenicity of Typhoid Vaccines as Related to Content of Vi Antigen*

Vaccines of *Salm. typhi* 58			Protective potency in mice challenged intracerebrally†		
Product	Antigens present	Method of preparation	Immunizing dose (ml.)	Survivors / Total	ED_{50} (ml.)
No. 1*	O	Heat-killed, phenol-preserved	0·4	3/18	—
			0·1	2/20	
No. 2	O+Vi	Heat-killed, phenol-preserved	0·4	14/23	0·17
			0·1	12/27	
			0·025	5/18	
No. 3	O+Vi	Acetone-killed and dried	0·1	16/25	0·04
			0·025	11/24	
			0·006	6/22	

* Prepared from the " O " variant of strain 58. Products No. 1 and 2 were the same vaccines employed previously (Landy, 1953), while product No. 3 was similar, but not identical.
† Challenge: 50,000 *Salm. typhi* Ty2: 15 control mice were injected with 0·5 ml. saline and challenged. None survived.

vaccine (No. 1) was non-protective, while vaccine No. 2 prepared from a strain containing Vi and O antigens, even though it had been subjected to heat-phenol treatment, was protective. The dehydrated vaccine (No. 3) also prepared from the Vi + O strain and presumed to contain essentially unaltered Vi antigen was more effective by a factor of > 4. The following tabulation shows that in a general way, these results are similar to those obtained with the same products in the protection test employing intraperitoneal challenge, except that differences in potencies appear greatly magnified in the latter procedure.

Vaccine	Relative protective potencies in mice challenged	
	Intraperitoneally	Intracerebrally
No. 1	0·08	Too small to measure
No. 2	1	1
No. 3	81	>4

Protective potency of Vi-containing organisms.—It was of interest to compare the protective activities of Vi-containing organisms from several genera against intracerebral infection of mice with *Salm. typhi*. For this purpose acetone-dehydrated organisms of Vi strains of *Bact. coli*, *Bact. ballerup* and *Salm. typhi* were employed. The results of this experiment, given in Table II, show that all Vi strains were protective. *Bact. coli* was the most active, *Bact. ballerup* only slightly less so, while the serologically rough Vi I strain of *Salm. typhi* was the least active. These results correspond well with the quantity of Vi antigen isolated from these organisms by Webster *et al.* (1955) who demonstrated that this strain of *Bact. coli* was a much richer source of Vi antigen than *Salm. typhi* strains.

TABLE II.—*Immunogenicity of Vi Organisms*

Acetone-dehydrated bacilli	Immunizing dose (μg.) 10	1	0·2	0·04
Bact. coli 5396/38 (Vi)	—	20/40*	16/40	10/40
Bact. ballerup 7851/39 (Vi)	—	23/39	16/40	4/40
Salm. typhi (Vi I)	12/24	4/26	—	—

* Survivors/total number of mice injected. Among 38 mice inoculated with saline only, there were no survivors.

Protection afforded by isolated antigens.—Since the Vi and O antigens have been isolated from *Salm. typhi* and serologically related organisms, it was possible to examine the immunity induced by these substances in the absence of other bacterial components. Groups of mice were injected intraperitoneally with Vi antigen from *Bact. coli* and, for purposes of comparison, other mice were inoculated with the O antigen derived from *Salm. typhi*. Six days later both groups were challenged intracerebrally with *Salm. typhi* Ty2. The results (Table III) show that the isolated Vi antigen is highly effective in protecting against intracerebral challenge in contrast to absence of protection by the O antigen, the other major antigenic component of *Salm. typhi*. In another test, groups of 60 mice each were injected intraperitoneally with 1 μg. of either purified Vi antigen (coli) or acetone-dehydrated *Bact. coli* (Vi strain) and challenged intracerebrally 6 days later. Eighty-eight and 78 per cent respectively were protected, as compared with no survivors in a control group of 40 mice.

TABLE III.—*Immunogenicity of Isolated Antigens*

Antigen	Immunizing dose (μg.) 25	5	1	0·2	0·04	0·008	0·0016
Vi (from *Bact. coli* 5396/38)	—	—	31/40*	24/40	17/40	5/40	0/40
O (from *Salm. typhi* O–901)	0/20	0/20	0/20	—	—	—	—

* Survivors/total number of mice injected. Result in controls receiving saline only 0/40.

Rate of development of immunity.—To determine the time required for development of specific protection against challenge, groups of mice were inoculated intraperitoneally with 1 μg. of isolated Vi antigen at intervals ranging from 6 days to 12 hr. before intracerebral challenge, and all groups were challenged at the same time. This experiment provides evidence (Table IV) that a rather high

TABLE IV.—*Rate of Development of Active Immunity following Administration of Isolated Vi Antigen*

Experiment	Days elapsed between intraperitoneal injection of Vi antigen and intracerebral challenge 6	3	2	1	1/2	0*	Controls (saline)
1	19/25†	20/25	17/25	5/23	4/23	1/25	0/25
2	18/20	15/20	14/20	4/20	1/20	—	0/19

* Antigen mixed with challenge and inoculated intracerebrally.
† Survivors/total number of mice injected.

degree of immunity is developed as early as 2 days following injection of purified Vi antigen. It also appears that a small but significant proportion of the mice respond in an even shorter time, as is evidenced by approximately 20 per cent survivors in groups inoculated 24 hr. before challenge.

Protection afforded by immune sera.—Experiments were designed to determine the extent to which the levels of specific antibody developed in mice in response to active immunization accounted for the observed protection against intracerebral challenge. Groups of mice were injected intraperitoneally with 1 μg. of either isolated Vi antigen (coli), dehydrated *Bact. coli* organisms, or dehydrated *Salm. typhi* O–901. The animals were exsaguinated 6 days later, the time interval at which immunized animals ordinarily were challenged. These sera, therefore, reflect the levels of circulating specific antibody present in comparably immunized mice at the time of challenge. The protective potency of these sera was ascertained in passive protection tests in which groups of mice were given graded doses of serum. Table V shows that mouse Vi antisera protect passively and that this protection is related to the dose administered. On the other hand, the sera of normal mice provide no protection against death nor do the sera of mice inoculated with typhoid O bacilli.* The antibody developed in response to either isolated Vi antigen or to the intact Vi-containing organism is therefore probably sufficient to account for the protection afforded by these products in active immunization tests.

TABLE V.—*Passive Protection Afforded by Specific Antisera*

Antisera								Controls	
		Titre		Dose of antiserum (ml.)				Normal serum	
Produced in	Developed against	Vi*	O†	0·2	0·1	0·04	0·02	(0·2 ml.)	Saline
Mice	Vi antigen (coli)	60	Neg.	9/17‡	—	4/18	1/19	0/20	1/19
	Bact. coli 5396/38 (Vi)	30	Neg.	10/19	—	4/18	0/19		
	Salm. typhi O–901	Neg.	Neg.	0/14	—	—	—		
Rabbits	*Bact. ballerup* (Vi)	1920	Neg.	17/20	—	13/20	—	0/20	0/20
	Salm. typhi O–901	Neg.	5000	—	1/20	—	0/20		

* Vi haemagglutination test.
† Bacterial agglutination test.
‡ Survivors/total number of mice injected.

Passive protection tests were also conducted with antisera prepared by immunization of rabbits with the Vi strain of *Bact. ballerup* or the O-901 strain of *Salm. typhi*. Here, too, the Vi antiserum was markedly protective while O antiserum of high titre failed to protect against intracerebral challenge.

The course of infection in immunized and control mice

Bacteriology.—In order to evaluate the effects of active immunization on the reaction of the host to intracerebral challenge, groups of 80 mice each were given

* It will be noted that mice receiving typhoid vaccine failed to produce demonstrable O antibody (Table V). The failure of this species to develop antibody in response to injection of O antigen in the form of either vaccine or purified substance has been observed in this laboratory on numerous occasions (Landy *et al.*, 1955). Moreover, Miller, Clark and Dierkhising (1951) have also noted that significant levels of O agglutinins (1/10 or over) were never obtained in mice 7–12 days after a single injection of various typhoid vaccines, even when these were administered in substantial dosage. In contrast, these workers reported that Vi agglutinins could invariably be detected in this species 7–12 days after a single injection of the same typhoid vaccines.

an immunizing injection intraperitoneally of 1 μg. of acetone-killed and dried *Bact. coli* or 1 μg. of the purified Vi antigen derived from this organism, while control animals received saline. Six days later all animals were challenged intracerebrally in the usual manner. According to a predetermined schedule, one-half of each group of animals was set aside for mortality data and one-half for bacteriological determinations. Five mice from each of these groups were removed at daily intervals and sacrificed for the determination of viable count in the entire brain, and dissemination of bacteria in the spleen and liver. It is apparent from the results of these tests, given in Table VI, that the response of the host to stimulation with the Vi antigen results in an effective suppression of bacterial multiplication following challenge. In the brain of immunized mice, there occurs some initial multiplication of the challenge, which appears to be limited at most to several multiples of the inoculum, and these numbers rapidly diminish to very low levels and subsequently disappear. On the other hand, in the brain of unprotected mice there occurs early massive increase in numbers which reaches high levels, maintained until death. The contrast in the reactions to challenge of immunized and unprotected mice is depicted graphically in Fig. 1.

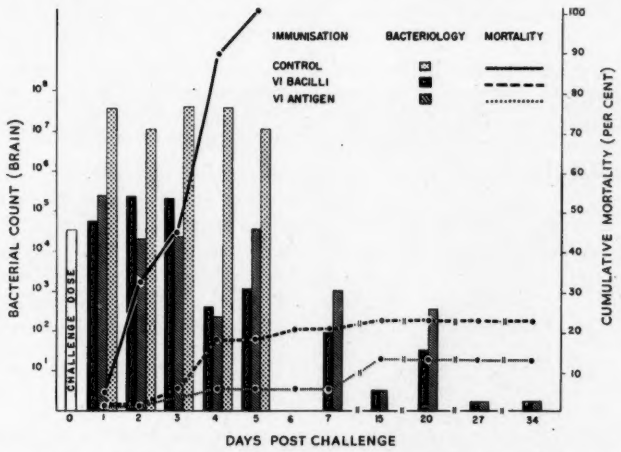


FIG. 1.—Course of intracerebral typhoid infection in immunized and control mice.

The numbers of bacilli found in spleen and liver varied from one interval to another in all groups during the first 3 days. Significant differences in numbers were not apparent in the 3 groups until the 4th and 5th days, at which time the numbers of bacilli in the organs of the control mice were considerably higher. Shortly afterwards, the liver and spleen of protected animals became organism-free.

Histopathology.—Correlation between histopathological findings and clinical course of the intracerebral infection, as modified by varying types of immunization of the host, was investigated in two principal sets of experiments. In the first, groups of mice immunized with Vi antigen were sacrificed 14 days after intracerebral challenge. In mice actively immunized with Vi antigen, in doses ranging from 0·008 to 5 μg. even as little as 0·008 μg. resulted in a remarkable

TABLE VI.—*Bacterial Multiplication and Mortality in Mice Inoculated Intracerebrally with* S. typhi *Ty*2. *Comparison of Immunized and Control Animals.*

		Vi antigen*		Vi bacilli†		Controls (saline)	
		Mortality‡	Bacterial count§	Mortality	Bacterial count	Mortality	Bacterial count
Days after intracerebral inoculation (40,000 organisms)	1	—	260,000	—	77,500	4	50,000,000
	2	—	26,000	—	300,000	28	5,000,000
	3	—	28,000	5	250,000	12	50,500,000
	4	5	300	12·5	530	44	50,500,000
	5	—	55,000	—	1,000	12	10,000,000
	6	—	—	2·5	—		
	7	—	1,000	—	100		
	15	7·5	5	2·5	5		
	20	—	500	—	50		
	27	—	0	—	0		
	34	—	0	—	0		
Total mortality		12·5		22·5		100	

* Isolated from *Bact. coli* 5396/38.
† Acetone-dehydrated *Bact. coli* 5396/38.
‡ Daily mortality in per cent.
§ Each figure represents the average of separate viable counts made on the brains from 5 mice.

absence of any significant histological alterations in those which survived (12·5 per cent) (Fig. 2). In contradistinction, unprotected control mice succumbed to the infection within one week from suppurative, necrotizing meningoencephalomyelitis with abscess formation.

The protection afforded by immunization with Vi antigen was not absolute. An occasional mouse had a suppurative ependymitis, the lateral and third ventricles being distended with pus. It should be pointed out that these animals did not appear clinically ill. Their subarachnoid space contained only a slight amount of inflammatory exudate, and the brain parenchyma appeared uninvolved. These findings would indicate a partial protection of the animals with clearing of any infection from the subarachnoid space and brain tissue proper and persistence

EXPLANATION OF PLATES

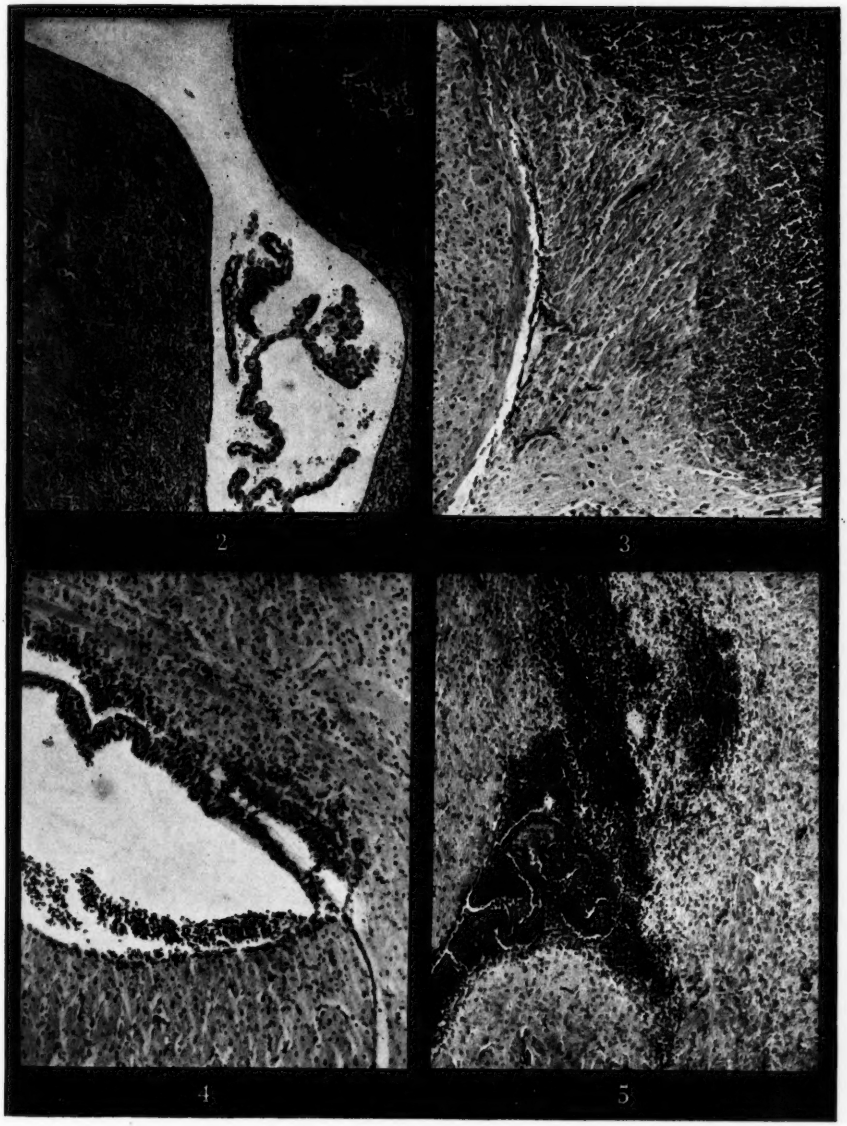
FIG. 2–5.—Sections of mouse brain, embedded in paraffin and stained with haematoxylin–eosin.

FIG. 2.—70 × Immunization: 0·008 μg. Vi antigen, challenge dose: 40,000 organisms, sacrificed 14 days post-challenge. Note absence of abnormal findings in choroid plexus, ependymal lining, midbrain (left) and cerebellum (right).

FIG. 3.—55 × Immunization: 0·5 ml. of commercial T.A.B. vaccine, challenge dose: 40,000 organisms, sacrificed 14 days post-challenge. Note filling of distended ventricle (on right) with purulent exudate. The ependymal lining is destroyed and the inflammatory reaction extends into the subependymal white matter paralleling the outline of the ventricle. The intra-cortical extension of the inflammatory reaction is limited.

FIG. 4.—70 × Immunization: 1 μg. of Vi antigen, challenge dose: 50,000 organisms, sacrificed 3 days post-challenge. Note evidence of internal hydrocephalus. There is an acute ependymitis involving also the choroid plexus. The inflammatory reaction is confined to the ventricle and the immediately adjacent subependymal white matter.

FIG. 5.—55 × Unprotected control animal sacrificed 3 days following challenge with 50,000 organisms. Note purulent ependymitis; elements of choroid plexus are completely surrounded by purulent exudate. The outline of the ventricle is indistinct and the ependymal lining is destroyed. There is a spreading suppurative encephalomyelitis.



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of the infection in a blocked ventricular system. With such exceptions, immunization with Vi antigen gave full protection over the range tested.

Mice passively immunized with 0·5 ml. typhoid O antiserum intraperitoneally did not survive the 14-day period of observation ; they succumbed to an intense meningo-ependymitis, the ventricles being distended with pus. There was also an early encephalomalacia of the adjacent subependymal brain tissue, secondary to the lesion in the ventricles. Despite massive multiplication of organisms in the subarachnoid space and particularly in the ventricles there was no significant bacterial invasion of the brain tissue proper or brain abscess formation, as seen in unprotected controls. Their absence in these O-immunized animals suggests a partial protection of the animal with what amounts to an erection of a " brain-cerebrospinal barrier ". This term is used in a descriptive sense only and it is not implied that such a barrier actually exists. It could also be argued that brain parenchyma of the immunized mouse is an unfavourable environment for *Salm. typhi*. Immunization with 0·5 ml. of commercial typhoid vaccine produced essentially an identical pattern. Such animals showed a massive suppurative ependymitis with distension of the ventricular system by pus and secondary focal encephalitis of subependymal brain tissue. There was also a slight to moderate meningitis (Fig. 3). Most of these animals died within the 14-day period of observation. In contrast, active immunization with 0·1 ml. of acetone-dehydrated typhoid vaccine, or passive immunization with 0·2 or 0·04 ml. of rabbit Vi antiserum resulted in full protection and minimal histological changes which were practically within the range of normal.

In a second set of experiments designed to elucidate further the effect of immunization with Vi antigen in altering the course of an intracerebral infection, two groups of mice were sacrificed 1, 2, 3, 4, 5, and 8 days following challenge with 50,000 organisms. One group had been actively immunized with 1 μg. of Vi antigen before challenge while the other was passively protected by 0·5 ml. of rabbit Vi antiserum. The histological findings for each time interval were so similar that it was not possible to differentiate the active from the passively immunized mice on the basis of tissue changes. Either form of Vi-immunizing agent, active or passive, resulted in a marked amelioration of the inflammatory response to intracerebral challenge. At the 24-hr. interval very little difference could be observed between unprotected control and immunized animals. However, by 48 hours differences in these groups became apparent. Both groups of immunized animals showed only a slight progression of the inflammatory reaction while unprotected controls showed a distinct advance in its intensity. At the 72-hr. interval the inflammatory reaction had levelled off in the immunized animals (Fig. 4) while it continued to progress in the controls (Fig. 5). In the immunized animals the reaction remained stationary at the 96-hr. interval, clearing thereafter, but residual effects were still clearly recognizable on the 8th day, by which time unprotected controls had succumbed.

DISCUSSION

In mouse protection tests employing intraperitoneal challenge with virulent *Salm. typhi*, earlier work has clearly shown that the Vi antigen is the essential immunizing component. The present study extends those observations by showing that this antigen likewise is of critical importance for protection against

intracerebral infection. The similarity of results obtained by the two methods probably is more than coincidental. Rather, the evidence leads to the conclusion that *only* with Vi antigen or antibody is it possible to immunize mice effectively against fatal infection with typical virulent typhoid bacilli.

Among the various findings reported here, the resistance of immunized animals to intracerebral challenge with many multiples of the M.L.D. is in itself strong evidence for the efficacy of the Vi antigen. The effectiveness of this immunity to intracerebral challenge is even further emphasized by the bacteriological and histopathological sequence of events in the brains of immunized and control mice. Examination of these changes reveals that in immunized mice, only limited multiplication of the challenge inoculum occurs, and that the relatively small number of bacilli surviving in the brain are subsequently eliminated. These data are in good agreement with the character of the changes in the brain. Since it was also shown that the sera of Vi-immunized mice afforded passive protection against intracerebral challenge, presumably the restriction of bacterial growth and the prevention of tissue damage are attributable in both instances to Vi antibody. This may be considered evidence for the specific humoral nature of the immunity engendered by the Vi antigen. It follows that in the mouse antibodies reach the brain in contrast to other animal species where the " blood-brain barrier " effectively prevents their passage. The classical example of the latter situation is meningococcal meningitis in man in which serum was effective only when administered intrathecally.

To the extent that the Vi antigen exerts the dominant protective action, the results obtained in this study generally confirm earlier work with intraperitoneal challenge. However, one important difference between the two infection procedures was noted. In this report, it was shown that the typhoid O antigen alone was quite without effect in preventing death. On the other hand various workers (Topley, Raistrick, Wilson, Stacey, Challinor and Clark, 1937 ; Henderson and Morgan, 1938) and a study in this laboratory as well (Landy *et al.*, 1955) have previously shown that in mice challenged intraperitoneally, O vaccines or antigen do provide protection of varying degree. However recent reports have indicated that these effects by O antigen are largely of a non-specific nature ; *i.e.*, any of a variety of somatic antigens (Landy and Pillemer, 1956 *a*, *b*) or vaccines of Gram-negative bacteria (Field, Howard and Whitby, 1955) induced an early rise in non-specific resistance to experimental infection with Gram-negative pathogens, including *Salm. typhi*. Unpublished experiments by one of us have shown that the induction of a non-specific rise in resistance by antigens of smooth and rough Gram-negative bacterial species (lipopolysaccharides) may be limited to infections initiated by the intraperitoneal or intravenous routes, since treatment of mice with these substances is essentially without effect on infection produced by intracerebral inoculation. It would thus appear that the response of mice infected by the intraperitoneal route is affected markedly by non-specific factors, while the resistance of intracerebrally challenged mice is specific.

Study of histological changes during the course of intracerebral infection in immunized and unprotected mice has revealed a considerable range of alterations which correlated with and augmented the clinical and bacteriological data. In particular, histological study revealed a range of tissue responses in surviving animals as well as those which eventually succumbed and thus defined the characters of the inflammatory response, particularly as it is modified by active

or passive immunization. These finer gradations of response are not discernible when the sole criterion of protection is survival or death.

The infection established in mice by the intracerebral inoculation of *Salm. typhi* has attributes considered characteristic of lethal bacterial infections, such as a small infective dose, a well defined incubation period, extensive multiplication of the invading organism, appearance of symptoms of increasing severity, progressive development of brain lesions, and fatal termination. These characteristics of the intracerebral infection, taken together with the specificity of the immunological response, overcome the major objections to the intraperitoneal procedure. The use of the intracerebral technique may therefore provide a more meaningful evaluation of typhoid vaccines and other antigenic components of possible prophylactic value.

The study of experimental infection and immunity in pertussis formerly presented a comparable problem ; *i.e.*, to determine the most appropriate *in vivo* method for measuring the potency of immunizing agents. It is noteworthy that in the evaluation of these agents, protection afforded to mice against intracerebral challenge with *Haemophilus pertussis* and protection against natural infection in human beings were recently shown to be correlated (Report to the Medical Research Council, 1956). Obviously, the reliability of laboratory potency tests of typhoid prophylactics will be established with comparable certainty only when vaccines and isolated antigens are evaluated not only in mice but also in terms of protecting man against typhoid fever.

SUMMARY

In a study of the factors protective against intracerebral infection of mice with *Salm. typhi* it was shown that, of the two major antigenic components of this organism, the isolated Vi antigen was highly protective against fatal infection while the purified O antigen exerted no such effect. Tests with typhoid vaccines yielded essentially similar results ; *i.e.*, vaccines with the most Vi antigen provided the best protection. Passive tests with sera of immunized rabbits and mice showed that Vi antibody protected against this infection while O antibody was relatively ineffective.

Study of the course of the intracerebral infection in immunized and control mice disclosed that in mice receiving purified Vi antigen there was an effective suppression of bacterial multiplication in the brain. In contrast, untreated mice displayed an early massive increase in numbers of bacteria. Immunization with O antibody, despite failure to prevent death, resulted in a significant alteration of the histological pattern. Mice immunized with either Vi antigen or antibody exhibited only slight to moderate transient tissue damage and no evidence of progressive meningoencephalitis, the picture characteristic of this infection in unprotected animals.

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PHAGOCYTOSIS OF HEAT-DENATURED HUMAN SERUM ALBUMIN LABELLED WITH ^{131}I AND ITS USE AS A MEANS OF INVESTIGATING LIVER BLOOD FLOW

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WHEN protein solutions are heated colloidal aggregates are formed (Neurath, Cooper and Erickson, 1941; Cooper and Neurath, 1943). The degree of aggregation and the size of the aggregates depend, for a given protein, on the concentration, the temperature, the pH and the salt concentration (Joly and Barbu, 1949). In previous experiments concerning the protein aggregates of heated whole serum (Benacerraf, Halpern, Stiffel, Cruchaud and Biozzi, 1955; Biozzi, Halpern, Benacerraf, Stiffel and Mouton, 1956) we observed that the cells of the reticulo-endothelial system (R.E.S.) phagocytize the complexes of globulin and albumin (C.A.G.) which can be isolated from heated serum. The kinetics of blood clearance of C.A.G. tagged with ^{131}I follows the laws established for other colloids phagocytized by the R.E.S. (Biozzi, Benacerraf and Halpern, 1953; Benacerraf, Stiffel, Biozzi and Halpern, 1954; Halpern, Benacerraf, Biozzi and Stiffel, 1954). The blood concentration decreases exponentially with respect to time and the rate of clearance is inversely proportional to the dose of C.A.G. when enough colloids are injected.

In this study we have verified that the R.E. cells phagocytize the aggregates of human serum albumin (C.A.). The sensitivity of the radioactive techniques allows the study of the blood clearance of very small doses of C.A.^{131}I, while the lack of toxicity and the stability in the blood of the protein permit the injection of high doses for the investigation of R.E. phagocytic activity. We have observed that there is in every animal species a critical dose of this protein below which the rate of clearance is constant and independent of the dose of colloid injected. In this range, C.A.^{131}I clearance can be used to measure liver blood flow as efficiently as the chromium phosphate technique (Dobson and Jones, 1952). A study has been made also of the efficiency of portal clearance of C.A.^{131}I in this dose range.

The breakdown of heat-denatured human serum albumin by the cells of the R.E.S. after it has been phagocytized was also investigated.

METHODS

Preparation and Iodination of Human Albumin Complex (C.A.)

The human serum albumin was supplied by the Centre National de la Transfusion Sanguine, available in a 25 per cent solution for clinical use. This solution contained mandelic acid 0·05 M as a stabiliser. A dilution containing 1 per cent albumin was prepared with

normal saline and the pH adjusted to 7. The optical density was then measured in the 550 mμ wave band in a 1 cm. square cell. Heating was carried out as described below until the optical density measured on aliquot samples was increased by 0·085 unit ± 10 per cent. The solution was heated at 70° in a water bath with constant shaking for 20 min.; the optical density was measured, and the temperature was brought up to 75°–80° and the optical density measured again every 5 min. until the required value was reached. This usually required 15–20 min. The solution was then immediately cooled below 50°. The times of heating, however, are not absolutely standardized and are controlled through optical density measurements because small changes of pH determine great changes in the effects of heating. This method was found to be more satisfactory than the use of buffered solutions for heating.

The albumin complex was then isolated by precipitation at its isoelectric point. After cooling to room temperature, the heated solution was acidified with N- or 0·1 N-HCl, until precipitation occurred. The supernatant was discarded after centrifugation and the precipitate washed several times with normal saline at pH 5·4 until the washings no longer contained material precipitable by 20 per cent trichloracetic acid. The albumin complex was then dissolved in normal saline by adding N- or 0·1 N-NaOH until pH 7·6. In this way solutions containing up to 30 mg. of protein per ml. can be prepared. The solution was left overnight in the refrigerator to ensure solubilization. It was then centrifuged and the insoluble fraction discarded. Merthiolate 1/5000 was added. It can be kept in the refrigerator at 4° for several months. Care must be taken not to let it freeze because the C.A. will then precipitate and become insoluble.

Iodination of C.A.

Iodination of C.A. with iodine containing ^{131}I was carried out by the method described for C.A.G. (Biozzi *et al.*, 1956). With this technique, the amount of iodine which was bound to the C.A. varied from 0·42 mg. to 0·72 mg. iodine/100 mg. protein. In order to obtain samples of C.A.^{131}I radioactive enough to allow the measurement of very small doses of the protein injected, several samples of C.A. were iodinated with considerably more ^{131}I; up to 2 millicuries of ^{131}I were used for 3 mg. of protein.

Methods of estimation of C.A.^{131}I

C.A.^{131}I was injected intravenously in a wide range of doses into mice, rats, guinea-pigs and rabbits. Measured blood samples, 0·025 or 0·05 ml., were drawn with a calibrated glass pipette from the retro-orbital venous plexus in mice, rats and guinea-pigs (Halpern and Pacaud, 1951) and from the ear vein in rabbits at standard times after the intravenous injections. The blood samples were spread on round filter paper 2·5 cm. diameter on glass slides and β-radioactivity measured with a Geiger Müller counter. After the animal had been killed and the liver, spleen, lung and kidney had been weighed, the γ-radioactivity of weighed samples of tissues was measured with a scintillating counter keeping the geometry constant. Samples of the injected material were measured in the same conditions.

Methods of estimation of chromium phosphate tagged with ^{32}P

Chromium phosphate containing ^{32}P was prepared by the usual technique by Dr. Noller. The blood clearance of small doses of this colloid was investigated in mice. Chromium phosphate tagged with ^{32}P was measured in blood samples through its β-radiations by the method used for ^{131}I. Chromium phosphate tagged with ^{32}P was measured in the tissues, after hydrolysis of the organs in concentrated NaOH: 98 per cent of the injected radioactivity was found in the liver.

Methods of estimation of small doses of carbon

Blood clearance of a very small dose of carbon was investigated in rats. Carbon suspension C11/1431 a (Gunther Wagner) in doses of 0·5 mg./100 g. was injected into rats intravenously. Estimation in the blood was carried out as follows: blood samples 0·10 ml. each, drawn from the retro-orbital venous plexus with a calibrated pipette washed with heparin were added each to 1·4 ml. of normal saline. The samples were centrifuged to get rid of the cells and the carbon concentration measured in the supernatant electrophotometrically.

RESULTS

Distribution of Injected C.A.^{131}I among the Various Organs

Table I shows the distribution of the injected radioactivity in mice, rats, guinea-pigs and rabbits for various doses of C.A.^{131}I. When very small doses are injected, the rate of clearance is very rapid and the animal can be killed a few min. after the injection. In this case 90 to 100 per cent of the injected radioactivity is detected in the liver and spleen. The liver contains nearly all the injected radioactivity, showing that for very small doses of colloids the phagocytic efficiency of the liver is greater than that of the spleen. When higher doses of C.A.^{131}I are injected the percentage found in the spleen increases as expected from our experience with carbon particles. However, only a fraction of the injected radioactivity can be recovered in the organs analysed; this can be as little as 43 per cent when 5 mg./100 g. of C.A.^{131}I are injected. In the latter experiments, the animals were killed 20–70 min. after injection and these differences are probably due to the rapid metabolism, release into the blood and mainly renal excretion of the C.A.^{131}I. A residual blood radioactivity of 7 per cent of the injected dose can be found.

To substantiate these facts we injected a group of 40 mice with 0·25 mg./100 g. of C.A.^{131}I, a dose nearly completely cleared by the liver in a few minutes. The animals were killed at various times after injection and the radioactivity of the various organs measured as described (Fig. 1). C.A.^{131}I is attacked very rapidly by the liver. From 6 to 30 min. over 50 per cent of the ^{131}I is released from the liver.

Blood clearance of C.A.^{131}I in mice

A group of 85 mice were injected intravenously with various doses of C.A.^{131}I ranging from 0·025 to 10 mg./100 g. Several different preparations were used with similar results. The rate of blood clearance was measured and at the end of the experiments the animals were killed, and the liver and spleen weighed in the wet state. The ratio, *W/Wls*, of the weights of the liver and spleen to body wt. was calculated.

In Fig. 2 we present typical experiments. For a very small dose of C.A.^{131}I, 0·025 mg./100 g., the blood clearance follows an exponential function with respect to time. The phagocytic index K which is the rate of clearance can therefore be calculated from equation 1 :

$$\frac{\log C_1 - \log C_2}{T_2 - T_1} = K \text{ (decimal logarithm).}$$

When much larger doses of C.A.^{131}I are injected, there remains at the end of the experiment a residual blood radioactivity (Table II), due to the escape of ^{131}I into the blood when it is released from the R.E. cells. When the values of each point of the clearance curve are corrected by subtracting the residual blood radioactivity, the rate of clearance follows also an exponential equation and the phagocytic index K can be calculated (equation 1). This procedure has been followed in the calculation of K when necessary.

TABLE I.—*Distribution of C.A.*^{131}I *after Intravenous Injection in Mouse, Rat, Guinea-pig and Rabbit*

	Dose of C.A.^{131}I.	Interval after injection (minutes).		Liver.	Spleen.	Kidney.	Lung.	Blood.	Total
Mouse	0·25 mg./100 g.	6	Per cent of injected dose	98·0	2·0	0·75	0	—	100·75
			Per cent/g.	77·0	10·0	2·3	—	—	—
	2·5 mg./100 g.	22	Per cent of injected dose	62·0	2·7	2·0	1·2	7·5	75·4
			Per cent/g.	51·5	13·5	7·2	5·3	—	—
Rat	0·25 mg./100 g.	7	Per cent of injected dose	93·0	1·9	1·2	0·1	2·0	98·2
			Per cent/g.	19·5	4·2	1·1	0·1	—	—
	5 mg./100 g.	60	Per cent of injected dose	34·0	1·0	1·1	0·8	7·0	43·9
			Per cent/g.	6·7	2·5	1·0	0·6	—	—
Guinea-pig	0·1 mg./100 g.	6	Per cent of injected dose	91·0	1·0	0·25	1·0	—	93·25
			Per cent/g.	7·9	1·7	0·08	0·3	—	—
	2 mg./100 g.	30	Per cent of injected dose	61·4	1·2	1·5	0·4	5·0	69·5
			Per cent/g.	4·6	3·2	0·34	0·16	—	—
	5 mg./100 g.	70	Per cent of injected dose	36·0	0·8	1·5	0·5	6·0	44·8
			Per cent/g.	2·7	1·6	0·4	0·19	—	—
Rabbit	0·1 mg./ 100 g.	.	Per cent of injected dose	88·0	1·7	0·4	0·3	2·0	92·4
			Per cent/g.	1·14	1·94	0·03	—	—	—

The figures are average values in 3 or 4 animals per dose.

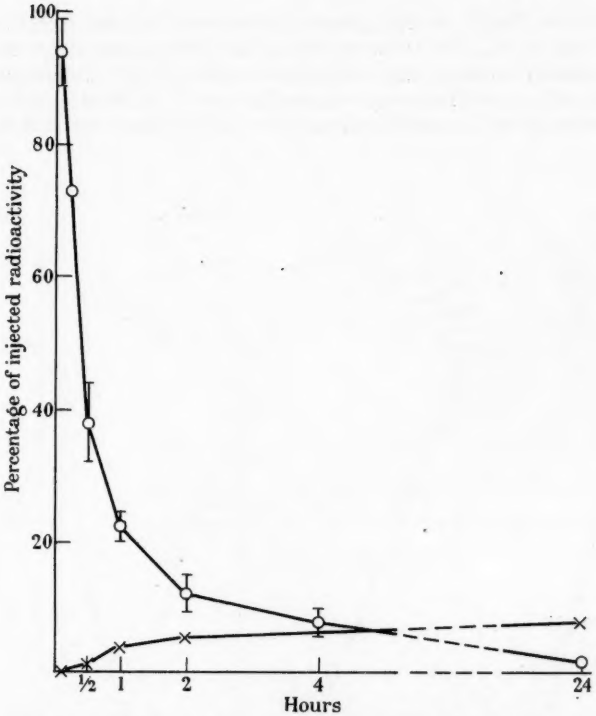

FIG. 1.—Measure of radioactivity of the liver and the thyroid in mice injected with 0·25 mg. per 100 g. of C.A.^{131}I at various times following the injection ○———○ = liver. ×———× = thyroid.

In Table II we present for each dose of C.A.^{131}I the mean phagocytic index K, the residual blood radioactivity, the mean value of W/Wls and the corrected phagocytic index α calculated from equation 2:

$$\alpha = \frac{W}{Wls}\sqrt[3]{K}$$

TABLE II.—*Blood Clearance of Various Doses of C.A.^{131}I in Mice*

C.A.^{131}I (mg./100 g.).	Number of animals.	Phagocytic index K.	Average residual blood radioactivity per cent injected dose.	$\frac{W}{Wls}$.	Corrected index α.
0·025	5	0·400±0·076	2·5	19·4	—
0·125	5	0·388±0·082	3	16·2	—
0·250	5	0·386±0·110	3	15·9	—
0·500	4	0·285	3	19·8	13·0
1·25	4	0·150	4	18·0	9·5
2·5	39	0·084±0·031	4	18·1	7·7±0·65
5·0	16	0·042±0·016	6·5	18·4	6·5±0·4
10·0	6	0·027±0·019	9	17·3	4·9±0·5

In a previous study of the phagocytic activity of the R.E.S. with carbon particles (Biozzi *et al.*, 1953) we observed that in rats and mice there is a third power relationship between the phagocytic index K and the relative weight of the liver and spleen in the range where the rate of colloid clearance measured by K is inversely proportional to the injected dose. The corrected index α which

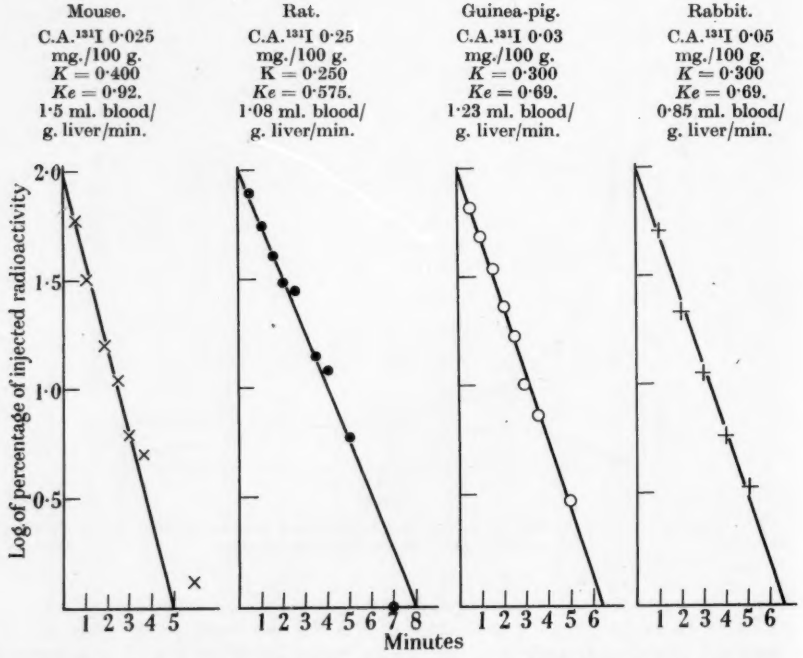

FIG. 2.—Rate of clearance from the blood of small doses of C.A.^{131}I in mouse, rat, guinea-pig and rabbit in the range of dose where the rate is maximum. Measure of " minimum liver blood flow ".

is a measure of tissue phagocytic activity presents much smaller standard deviations than K.

The standard deviations of K and α for doses of C.A^{131}I where $K \times D = ct$ are similar to those reported for carbon particles in mice (Biozzi, Benacerraf, Stiffel and Halpern, 1954). The standard deviations of α are also smaller than those of K. The C.A.^{131}I clearance measures therefore the same phagocytic activity of the R.E.S. as carbon clearance, and the calculation of α from equation 2 effects a real correction. For these reasons we have corrected the observed mean values of K with the help of equation 2, by choosing a standard average value of $W/Wls = 18{\cdot}3$ calculated from all our mice. In Fig. 3 we have plotted the variations of the corrected values of K with respect to the injected dose of

C.A.^{131}I. The data presented show that the rate of clearance of C.A.^{131}I is inversely proportional to the injected dose : $K \times D = ct$.

However there is a critical dose of C.A.^{131}I below which the rate of clearance is no longer a function of the dose and tends to become constant as it reaches its maximum value. In this range the only limiting factor is the blood flow through

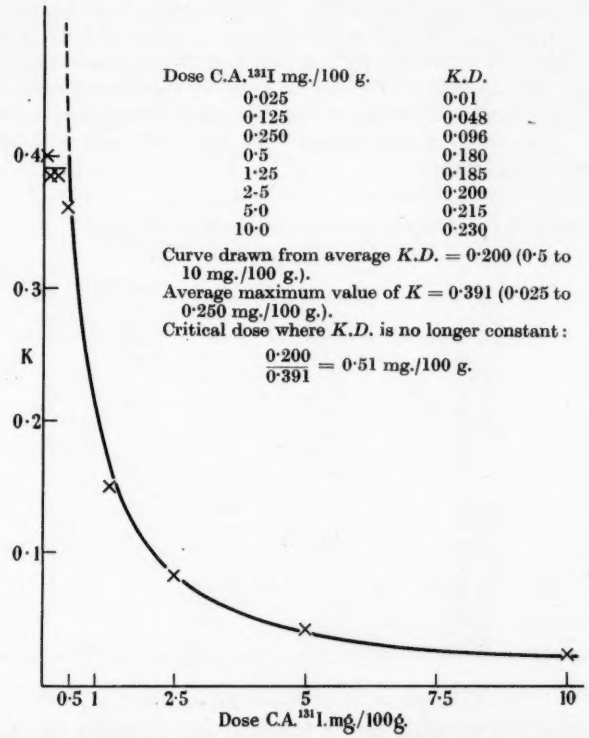

FIG. 3.—Variation of the phagocytic index K in relation with the dose of C. A.^{131}I in mice.

the R.E.S. and principally through the liver. The efficiency of colloid clearance in these organs reaches then a maximum value independent of the dose of colloid injected. The efficiency factor can be measured experimentally as we shall see later. It should be stressed therefore that the colloid clearance test measures the phagocytic activity of the R.E.S., only when a sufficient dose is injected, where $K \times D = ct$. The limits of this relationship should be established experimentally in each case. The critical R.E. dose can be found in any animal for a given colloid by measuring the maximum rate of clearance, with a very small dose and the rate of clearance in the range where $K \times D = ct$ (Fig. 3).

The critical R.E. dose is then calculated from equation 3 :

$$\text{critical R.E. dose} = \frac{K \times D}{\text{maximum value of } K}$$

Blood clearance of C.A.^{131}I in rats, guinea-pigs and rabbits

The blood clearance of C.A.^{131}I in rats, guinea-pigs and rabbits follows the same laws as in mice (Table III). The rate of clearance is exponential (Fig. 2), the value of K decreases directly with the dose so that $K \times D = ct$. There is a critical R.E. dose also below which the value of K reaches a maximum and no longer changes. The critical R.E. dose of C.A.^{131}I calculated from equation 3 is 0·43 mg./100 g. for the rat, 0·36 mg./100 g. for the guinea-pig and 0·19 mg./100 g. for the rabbit. The value of the critical R.E. dose will vary with the size of the liver and spleen as expected.

TABLE III.—*Blood Clearance of Various Doses of C.A.^{131}I in Rats, Guinea-pigs and Rabbits*

	C.A.^{131}I (mg./100 g.).	Number of animals.	Phagocytic index K.	Average residual radioactivity per cent injected dose.	$K \times D$.
Rats	0·012	3	0·325	1·5	0·0039
	0·05	6	0·341	2·0	0·017
	0·25	6	0·245	2·0	0·061
	0·5	3	0·268	2·0	0·134
	1·0	3	0·107	4·4	0·107
	2·5	5	0·062	5·2	0·155
	5·0	4	0·025	7·0	0·125
Guinea-pigs	0·03	5	0·299	0·0	0·0089
	2·0	4	0·053	5·0	0·106
	5·0	4	0·023	6·3	0·115
Rabbits	0·05	5	0·293	1·5	0·014
	0·10	5	0·280	1·1	0·028
	1·0	4	0·064	—	0·064
	2·5	4	0·018	—	0·045

The Measure of Liver Blood Flow with C.A.^{131}I

Below a critical R.E. dose of C.A.^{131}I, the rate of blood clearance attains a maximum value and the efficiency of clearance in the liver reaches a maximum. This technique can be applied to the measure of blood flow through the liver which for these doses absorbs nearly all the colloid injected.

The application of the colloid clearance test to the measure of liver blood flow has been introduced by Dobson and Jones (1952) with chromium phosphate containing ^{32}P. The principle of the method is as follows. If the liver completely clears the blood of the injected colloid, the specific rate constant Ke calculated from Naperian logarithms and equation 1 measures the fraction of the blood volume passing through the liver per unit time. The volume of blood flowing through the organ will be $Ke \times$ blood volume.

This method measures the true blood flow through the liver only if the efficiency of colloid clearance by the organ is 100 per cent, otherwise it gives a lower value which we call "minimum liver blood flow". This value has to be corrected

for the efficiency factor to measure true liver blood flow. It is essential to know the maximum efficiency of liver clearance of the colloid injected to measure the corrected liver blood flow. Dobson and Jones found an efficiency factor of 79 per cent in mice and 88 per cent in rabbits.

We have used the method of C.A.^{131}I clearance in the range of dosage where the rate of clearance is maximum to measure "minimum liver blood flow" in mice, rats, guinea-pigs and rabbits and we have compared the values observed with those obtained with other colloids. Table IV states the individual values of minimum liver blood flow in mice, measured with C.A^{131}I in the dose range where K is constant, from 0·025 to 0·25 mg./100 g. The values of blood flow are expressed in ml./g. of liver/min. The minimum liver blood flow measured in 15 mice with C.A.^{131}I is 1·3 $\pm$ 0·29 ml./g./min. with considerable individual variation.

TABLE IV.—*Blood Clearance of C.A.^{131}I for Doses under 0·5 mg./100 g., where Rate is Maximum. Measure of Minimum Liver Blood Flow in Mice*

Dose C.A.^{131}I (mg./100 g.).	Body wt. (g.).	*Ke* Naperian log.	Liver wt. (g.).	Minimum liver circulation. (ml./g./min.).
0·025	24	1·06	1·150	1·65
	29	0·78	1·400	1·21
	24·5	0·71	1·350	0·97
	24	0·920	1·100	1·5
	26·5	1·12	1·200	1·86
0·125	24·5	1·04	1·400	1·37
	19·5	0·92	1·200	1·12
	24·4	0·76	1·100	1·27
	24·5	0·65	1·150	1·04
	21·0	1·10	1·250	1·39
0·250	24·4	1·15	1·300	1·62
	22·5	1·1	1·100	1·69
	19·0	0·94	1·200	1·09
	24·5	0·65	1·300	0·93
	20·5	0·6	1·150	0·80
Mean values	23·5	0·90	1·22	1·3 $\pm$ 0·29

Blood volume value of 1·5 ml./20 g. was assumed in the calculations. Mean value computed from 35 mice.

In Table V the values of minimum liver blood flow in mice calculated from chromium phosphate clearance are presented. The same blood volume 1·5 ml./20 g. was considered. The results are almost identical to those obtained with C.A.^{131}I. The "minimum liver blood flow" measured in 10 mice was 1·2 ml./g./min. Minimum liver blood flow in rats is stated in Table VI, in guinea-pigs and in rabbits in Table VII. The value of minimum liver blood flow was 1·13 $\pm$ 0·2 ml./g./min. in rats, in rabbits it was 1·15 $\pm$ 0·25 and in guinea-pigs 1·3 ml./g./min. "Minimum liver blood flow" thus differs little in these 4 animal species, and varies more between animals within a single species.

In rats the minimum liver blood flow was also calculated from the values of carbon clearance for the dose of 0·5 mg./100 g. (below the critical R.E. dose for this colloid). The results are identical to those obtained with C.A.^{131}I.

In order to calculate true liver blood flow in mice, rats, guinea-pigs and rabbits, the efficiency of C.A.^{131}I liver clearance has to be measured. Since the values of

Ke in mice for C.A.^{131}I are the same as those for chromium phosphate (Tables IV and V), the efficiency of clearance by the liver must be identical for these two substances in this animal species.

We have considered in mice an efficiency of clearance of C.A.^{131}I of 79 per cent reported by Dobson for chromium phosphate. In the other animal species the efficiency factor was measured as follows: Very small amounts of C.A.^{131}I, 5 to 10 μg. in 0·1 ml. were injected slowly (10 to 20 sec.) into the superior mesenteric vein, so that it would go through the liver before reaching the general circulation. This was done under local anaesthesia. The rate of clearance of C.A.^{131}I was measured. In this case the blood γ-radioactivity was measured in the well

TABLE V.—*Blood Clearance of Small Doses of Chromium Phosphate and Measure of Minimum Liver Blood Flow in Mice*

Dose chromium phosphate (ml./20 g.).	Body wt. (g.).	*Ke* Naperian log.	Liver wt. (g.).	Minimum liver circulation (ml./g./min.).
0·10	23·0	1·72	1·300	2·27
	21·5	0·50	1·100	0·73
	20·0	0·71	1·00	1·06
	19·0	0·62	0·850	1·04
	19·0	0·85	0·900	1·34
	22·0	0·69	1·050	1·08
0·25	24·0	0·76	1·300	1·05
	22·5	0·62	1·300	0·81
	27·5	1·15	1·450	1·63
	21·0	0·71	1·050	1·06
Mean values	21·8	0·833	1·130	1·2

Chromium phosphate 0·5 mg./ml.

TABLE VI.—*Blood Clearance of C.A.^{131}I for Doses under 0·5 mg./100 g. where Rate is Maximum. Measure of Minimum Liver Blood Flow in Rats*

Dose C.A.^{131}I (mg./100 g.).	Body wt. (g.).	*Ke* Naperian log.	Liver wt. (g.).	Minimum liver circulation (ml./g./min.).
0·012	104	0·828	4·500	1·38
	114	0·621	5·000	1·01
	103	0·805	5·000	1·19
0·050	131	0·943	6·200	1·43
	166	0·655	7·900	0·99
	132	0·855	6·300	1·28
	154	0·690	7·100	1·08
	161	1·04	8·300	1·45
	171	0·545	6·500	1·03
0·250	130	0·632	5·800	1·02
	144	0·565	7·100	0·83
	165	0·575	7·300	0·94
	135	0·552	4·750	1·13
	135	0·437	5·500	0·77
	135	0·621	5·200	1·16
Mean values	139	0·691	6·163	1·13±0·2

A blood volume value of 7·2 ml./100 g. was assumed in the calculation of liver blood flow. Mean value computed from 15 rats.

TABLE VII.—*Maximum Blood Clearance of C.A.*^{131}*I and Measure of Minimum Blood Flow in Guinea-pig and Rabbit*

Dose C.A.^{131}I (mg./100 g.).	Body wt. (g.).	*Ke* Naperian logarithm.	Liver wt. (g.).	Blood volume (ml./100 g.).	Minimum liver circulation (ml./g./min.).
Guinea-pig					
0·03	325	0·690	11·15	10·4	2·1
	325	0·747	11·35	5·6	1·2
	332	0·690	15·05	6·4	0·97
	330	0·805	12·40	6	1·28
	310	0·506	10·15	6·75	1·04
Mean values	324	0·687	12·02	7·0	1·3
Rabbit					
0·05	2·500	0·805	101·5	6·1	1·22
	2·100	0·414	65·0	6·7	0·89
	2·320	0·747	81·0	6·1	1·30
	2·920	0·713	96·5	5·6	1·20
	2·350	0·690	113·0	5·9	0·85
0·1	2·500	0·621	85·0	6·0	1·1
	3·250	0·736	116·0	8·3	1·7
	2·500	0·621	81·0	6·1	1·17
	2·300	0·437	65·0	6·7	1·04
	2·350	0·805	113·0	5·9	0·99
Mean values	2·500	0·659	91·7	6·3	1·15±0·25

Individual values of blood volume were considered in the calculation of liver blood flow.

scintillating crystal in order to increase the sensitivity of measurement. The curves were then extrapolated to 0 time. The initial blood concentrations obtained after injection in the mesenteric vein were compared with the concentrations at 0 time had the same dose of C.A.^{131}I been injected in the usual way in the systemic circulation. The ratio of the two concentrations at 0 time gives the efficiency factor of clearance of the colloid by the liver. Care was taken to inject amounts of C.A.^{131}I reaching the liver in a blood concentration where the rate of clearance is maximum.

The efficiency factor varies very little in the 3 animal species studied. The values observed are practically identical with those reported in the literature for chromium phosphate in the live animal by Dobson and Jones (1952) and in the rat liver perfused under physiological conditions by Brauer, Leong, McElroy and Holloway (1956).

TABLE VIII.—*Measure of Corrected Liver Blood Flow in Mice, Rats, Guinea-pigs and Rabbits*

	Minimum liver blood flow (ml./g./min.).	Efficiency factor (per cent).	Corrected liver blood flow (ml./g./min.).
Mouse	1·3	79 (Dobson & Jones)	1·64
Rat	1·13	84	1·35
Guinea-pig	1·3	77	1·68
Rabbit	1·15	82	1·4

In Table VIII the values of corrected liver blood flow for the 4 species studied are presented. Our results show that the amount of blood flowing through the liver per unit wt. varies little in these different species, although the values are somewhat larger in the mouse and the guinea-pig than in the rat and the rabbit.

DISCUSSION

The data reported establish that heat-treated human serum albumin aggregates are phagocytized by the cells of the R.E.S. with a high degree of efficiency, similar to what is observed with unstable preparations of chromium phosphate. Heat denaturation and aggregation of proteins render them readily absorbed by the R.E.S. in spite of the fact that they are soluble at a pH other than their isoelectric point.

The method of preparation and isolation of C.A.^{131}I described yields a product which is phagocytized homogeneously by the cells of the R.E.S. The CA.^{131}I follows all the laws of clearance of homogeneous colloids phagocytized by the cells of the R.E.S which makes it a valuable means of investigating phagocytic activity.

It should be emphasized again that to explore the phagocytic activity of the R.E.S. by the method of colloid clearance, a sufficient dose of the colloid has to be injected in the range where $K \times D = ct$. Below that dose the method is only blood flow-dependent, as only part of the R.E. cells are exposed to the colloid injected.

The use of chromium phosphate (Gabrielli, 1951 ; Heller, 1955) or colloidal gold (Barrow, Tullis and Chambers, 1951) to explore the phagocytic activity of the R.E.S. should therefore be criticized. The results obtained with these methods cannot be considered as a measure of R.E.S. phagocytic activity because doses of these colloids were injected which are cleared from the blood at a rate independent of the dose injected. Chromium phosphate or colloidal gold cannot be injected in the required dosage for R.E.S. exploration because of their instability in the blood and the toxicity of gold. Their particle size moreover cannot be well standardized. There are very few colloids which due to their lack of toxicity, particle size homogeneity and stability can be injected in the required dosage to explore the phagocytic function of the R.E.S. Carbon and C.A.^{131}I are two such colloids. We have used them to explore this function in the same animal species and we have obtained identical results with the two methods (Halpern *et al.*, 1954).

The comparative phagocytic activity of the R.E.S. of the mouse, rat, guinea-pig and rabbit measured by the constant $K \times D$ for each substance is also the same with both substances ; the mouse has the most active R.E.S. and the rabbit the least active.

In this study we offer a new method of measuring the specific cellular phagocytic capacity of the R.E.S. independent of blood flow. We have defined the " critical R.E. dose " as the dose of a colloid phagocytized by the R.E.S. above which $K \times D = ct$ and below which K is maximum. It is a specific measure of cellular activity. The " critical R.E. dose " can be calculated for any animal by measuring both the maximum value of K and a value of K in the range where $K \times D = ct$. When these measures are made on the same animal, the small dose should be injected first, because its saturating effect is negligible on the R.E.S. with respect to the large dose, while the reverse is not true.

The critical R.E. dose varies as expected with the size of the liver and spleen. The critical R.E. dose of C.A.^{131}I in mg./100 g. is 0·51 in the mouse for a $W/Wls = 18{\cdot}3$; 0·43 in the rat for a $W/Wls = 23{\cdot}5$; 0·36 in the guinea-pig for a $W/Wls = 24{\cdot}5$ and 0·19 in the rabbit for a W/Wls = 29. It confirms that the mouse has the most actively phagocytic system, and the rabbit the least, due mainly to differences in the size of the liver and spleen.

Our experience has shown that C.A.^{131}I can be used to measure liver blood flow as efficiently as chromium phosphate and colloidal gold (Vetter, Falkner and Neumayr, 1954). The measure of liver blood flow with C.A.^{131}I has shown that the amount of blood that flows through the liver per unit wt. varies very little in the different animal species studied, and varies more between individuals in a single species. Dobson and Jones, with the chromium phosphate technique, found smaller values of liver blood flow in the rabbit, and slightly higher values in the mouse. The efficiency factors of clearance are however identical for C.A.^{131}I and chromium phosphate in mice, rats and rabbits.

The speed with which C.A.^{131}I is broken down by the R.E.S. is also remarkable. This protein or other protein treated in the same way by heat and properly labelled, lend themselves to the exploration of the metabolic function of the R.E. cells both in the normal subject and under the effect of drugs or disease, due to the efficiency with which they are absorbed by the R.E.S. and metabolized.

SUMMARY

A method is described to prepare, isolate and standardize a complex of heat-denatured human serum albumin (C.A.) which, when injected intravenously, is phagocytized efficiently by the R.E.S. This protein has been labelled with ^{131}I and used to investigate the phagocytic activity of the R.E.S. in various animal species. The R.E. cells phagocytize this substance according to the same laws as apply to other colloidal suspensions. The blood clearance follows an exponential function; the rate of clearance is inversely proportional to the dose of colloid, when sufficient quantity of substance is injected. There is in every animal species a critical dose below which the rate of clearance is maximum and independent of the dose of colloid injected.

C.A.^{131}I can be used to investigate the phagocytic activity of the R.E.S. provided a dose of this protein is injected in the range where $K \times D = ct.$

Below this dose range, the study of C.A.^{131}I blood clearance can be applied to the measure of portal blood flow. A study of portal clearance for these small doses of C.A.^{131}I has been made to calculate the corrected liver blood flow. The values obtained indicate that the liver blood flow per unit weight is very similar in the various animal species studied.

A study was also made of the breakdown of this protein after it has been phagocytized, showing that ^{131}I is very rapidly released from the cells of the R.E.S.

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THE EFFECT OF β-SITOSTEROL ON CHOLESTEROL-INDUCED ATHEROMA IN RABBITS WITH HIGH BLOOD PRESSURE

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THE large rise in blood cholesterol levels following the feeding of cholesterol to various species of animal can be prevented by the addition of plant sterols to the diet. The most widely distributed of the plant sterols are the various sitosterols ; β-sitosterol which is found in cotton seed oil, tall oil and wheat germ oil, and γ-sitosterol, which is the principal sterol in soybean oil and a minor component of wheat germ oil and rye germ oil (Fieser and Fieser, 1949).

Peterson (1951) found that after the simultaneous feeding of cholesterol and soybean sterols to chicks, lower blood cholesterol levels were found than when cholesterol alone was fed. Pollak (1953) made the same observation in the rabbit using mixed plant sterols, and Hernandez, Peterson, Chaikoff and Dauben (1953) and Hernandez and Chaikoff (1954) showed a reduction in absorption of cholesterol in the rat when soybean sterols or β-sitosterols were added to a diet containing cholesterol. Peterson, Nichols and Shneour (1952) also showed that soybean sterols decreased the amount of cholesterol-induced atheroma in the chick, and both Pollak (1953), using mixed plant sterols, and Shipley (1955), using β-sitosterol, made similar observations in the rabbit.

The present experiment was designed to test whether plant sterols would retard the production of cholesterol-induced aortic atheroma in rabbits with high blood pressure, since such rabbits have been shown to develop considerably more atheroma than those with normal blood pressure (Bronte-Stewart and Heptinstall, 1954). It was thought that such rabbits with high blood pressure would provide a much more rigorous test of the efficacy of plant sterols in preventing cholesterol-induced atheroma in the rabbit.

The results showed that plant sterols do cause a considerable decrease in the amount of aortic atheroma ordinarily obtained in rabbits with high blood pressure.

MATERIALS AND METHODS

Male Chinchilla and New Zealand White rabbits weighing approximately 2·5 kg. were used, and a high blood pressure was produced by the removal of the right kidney, followed 2 weeks later by the application of a silver clip (bent in a U-shape with a compressing dimension of 0·55–0·65 mm.) to the left renal artery (Pickering and Prinzmetal, 1938). Blood pressures were recorded by means of an ear capsule (Grant and Rothschild, 1934) in a warm room with the animals sitting on an electrically-heated pad. When a satisfactory level of high blood pressure was reached, 100 mm. Hg being regarded as a satisfactory level, the rabbits were divided into 2 groups. The first group was fed on diet 18 of Bruce and Parkes (1946) impregnated with cholesterol as described previously (Heptinstall and Bronte-Stewart, 1954) so that every 100 g. of food contained 0·75 g. cholesterol. Each animal was allowed to consume 3·5–4·2 g. cholesterol per week and animals with larger appetites were fed food containing 0·5 g. cholesterol per 100 g. so as not to exceed the allowance.

This was found to be necessary since some animals if kept on the standard 0·75 per cent diet would have consumed their quota in 4 days and would have had to be put on a non-cholesterol diet for the rest of the week with consequent fall in blood cholesterol level. The second group was fed the same diet except that β-sitosterol was added in an amount equal to three times the amount of cholesterol. Cholesterol was added to one batch of pellets and β-sitosterol to another, and the two batches mixed together. This was to obviate any possible *in vitro* combination occurring between cholesterol and β-sitosterol. The resultant food mixture contained 0·75 g. cholesterol and 2·25 g. β-sitosterol per 100 g. A less concentrated preparation was made for animals with large appetites but the cholesterol-sitosterol ratio was maintained at 1 : 3. The weekly consumption was 3·5–4·2 g. cholesterol and 10·5–12·6 g. β-sitosterol. The rabbits were allowed unlimited water and were weighed and bled for serum cholesterol estimations once per week. Serum total cholesterol estimations were made in duplicate using the method of Pearson, Stern and McGavack (1953). Blood pressures were recorded twice weekly.

At the end of 8 weeks the animals were killed and post-mortem examination carried out. The aorta was removed, pinned out on cork and a tracing three times normal size made by means of a pantograph to show the distribution of atheroma. Areas of atheroma were divided into 2 grades, the first consisting of prominently raised plaques and the second of only slightly raised plaques. The areas of the atheromatous parts were measured by planimetry and after assuming the prominently raised plaques to be twice as severely affected as the others, a score was given to each aorta depending on the percentage area occupied by atheroma. A score of over 100 will therefore be possible if much of the aorta is occupied by the prominently raised plaques.

Histological preparations, both paraffin and frozen, were made from most organs and the weights of the liver, spleen, adrenals and carcass recorded.

RESULTS

In both groups the rabbits remained well during the entire experiment and showed either no change or slight increase in weight. All consumed the requisite amount of food each week.

Blood pressure

Table I shows the levels of the blood pressures in the two groups. It will be seen that the levels and the amount of rise are comparable in the two groups. The levels in both groups showed little fluctuation during the course of the experiment with the exceptions of rabbits 50 and 15 in Group I. These showed a fall of 10–15 mm. Hg during the last 2 weeks but in spite of this had mean pressures of 100 and 99 mm. Hg respectively.

Serum total cholesterol levels

Serum total cholesterol levels at 2-weekly intervals are shown in Table II. Animals of Group I, fed cholesterol only, showed a steep rise during the first 2 weeks and a more gradual rise during the next 2 weeks. Thereafter with an occasional exception they remained steady for the last 4 weeks of the experiment. Rabbit number 50 showed a poor response all along and no satisfactory explanation could be found for this. Animals of Group II, fed cholesterol and sitosterol, showed a similar steep rise during the first 2 weeks but reaching a very much lower level. Thereafter there was no constant pattern of behaviour, some falling, others remaining steady and others rising. Those showing a further rise never exceeded 650 mg./100 ml. at any time with the exception of rabbit number 70 hich reached a level of 920 mg./100 ml. towards the end of the experiment.

TABLE I.—*Aortic Atheroma, Blood Pressure, Rise in Pressure, Mean Serum Total Cholesterol and Adrenal/Carcass Wt. Ratios*

	Rabbit number	Grade of aortic atheroma (score)	Blood pressure (mm. Hg)	Rise in pressure (mm. Hg)	Mean serum total cholesterol over last 4 weeks (mg./100 ml.)	Adrenal wt. × 100 / carcass wt.
Group I	22	103	114	42	1450	0·070
	17	82	120	46	1110	0·042
	63	66	105	30	1590	0·074
	20	63	99	16	960	0·073
	31	41	111	33	1040	0·086
	48	40	100	28	990	0·051
	50	22	100	21	520	0·074
	15	15	99	13	1170	0·073
				Mean	1104±318	0·068±0·015
Group II	66	15	99	26	550	0·055
	34	9	114	34	500	0·026
	70	4	100	28	660	0·035
	68	3	123	47	220	0·037
	51	1	101	27	170	0·037
	14	0	103	17	400	0·054
	9	0	106	31	180	0·042
	42	0	101	20	130	0·067
				Mean	351±154	0·043±0·014

Group I fed cholesterol only.
Group II fed cholesterol + β-sitosterol.

TABLE II.—*Serum Total Cholesterol Levels (mg./100ml.)*

	Rabbit number	Weeks on diet: 2	4	6	8
Group I	22	400	700	2000	1730
	17	1060	1320	1030	1050
	63	910	1660	1680	1500
	20	780	1000	1010	1150
	31	660	1070	940	1070
	48	360	730	960	1300
	50	480	250	560	730
	15	560	1050	1170	1300
Group II	66	380	520	480	660
	34	350	270	650	500
	70	250	420	630	920
	68	230	260	220	170
	51	260	190	100	210
	14	230	300	430	460
	9	180	250	140	150
	42	160	270	80	40

Group I fed cholesterol only.
Group II fed cholesterol + β-sitosterol.

Taking the groups as a whole the levels of serum cholesterol were significantly higher in Group I at all stages of the experiment. The mean serum cholesterol levels for the last 4 weeks of the experiment are shown in Table I.

Aortic atheroma

The amount of aortic atheroma in both groups is shown in Table I, and it will be seen that the amounts in Group I are considerably higher than in Group II. In no rabbit of Group II did the amount of aortic atheroma exceed the lowest amount in rabbits of Group I, while in 4 of the rabbits on the cholesterol and sitosterol diet atheroma formation was completely or almost completely prevented. Fig. 1 and 2 demonstrate the areas of atheroma.

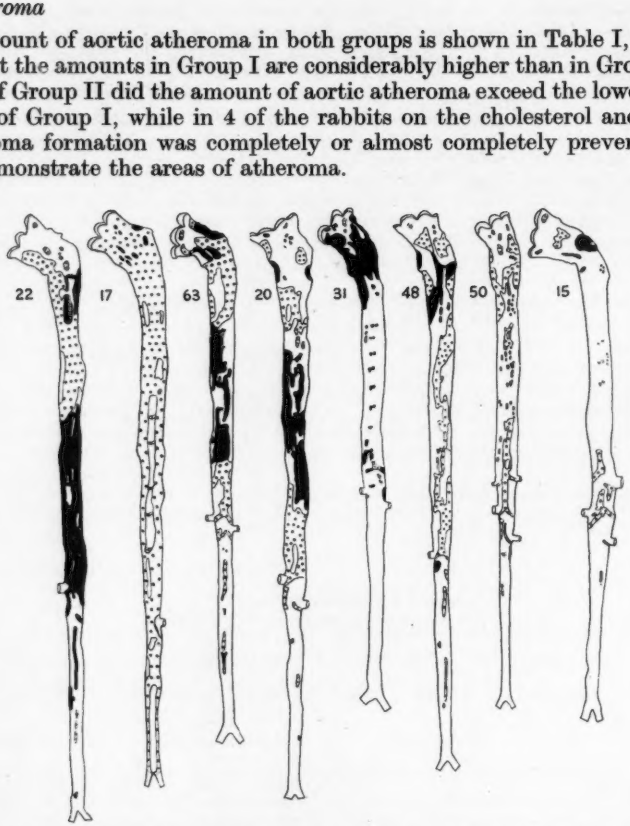

FIG. 1.—Amount of aortic atheroma in Group I (3/5 natural size). Dark areas represent prominently raised plaques and stippled areas slightly elevated plaques.

Visceral changes

Severe organ lipoidosis occurred in all rabbits of Group I, but this was conspicuous in only one rabbit, number 66, in Group II. All rabbits in Group II did however show some degree of cholesterol infiltration of liver, spleen and adrenal. The adrenal wt./carcass wt. ratio which serves as a crude measure of cholesterol absorption, was significantly lower in Group II than in Group I but was higher than the mean for rabbits on a normal diet. Arcus senilis was found with one exception in all rabbits of Group I but was present in no rabbit of Group II.

DISCUSSION

The plant sterol preparation used in this experiment contained over 95 per cent total sterols of which 90 per cent was β-sitosterol and not more than 10 per cent dihydro-β-sitosterol, and is referred to in this paper as β-sitosterol. The

results confirm that β-sitosterol decreases the level of circulating cholesterol when fed at the same time as cholesterol. Table II shows that the levels of serum total cholesterol are significantly lower in rabbits of Group II than in those of Group I. The failure of Dreisbach, Brown, Myers and Cutting (1953) to alter the level of blood cholesterol in the rabbit is probably due to the using of insufficient amounts of soybean sterol, the ratio of this to cholesterol in their diet being 1 : 1. Both Pollak (1953) and Valkema (1955) have shown that the greater the ratio of plant sterol to cholesterol, the greater is the capacity to prevent a rise in blood cholesterol. By using ratios of 7 : 1 both these workers found the absorption of cholesterol to be

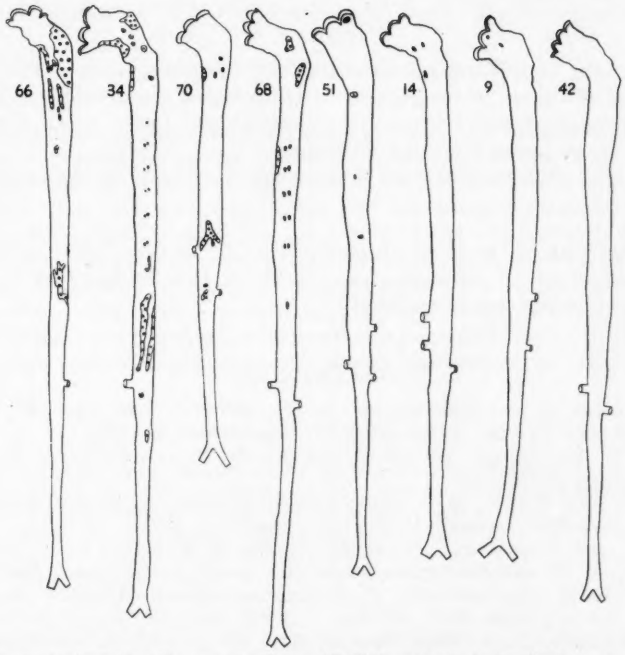

FIG. 2.—Amount of aortic atheroma in Group II (3/5 natural size). Dark areas represent prominently raised plaques and stippled areas slightly elevated plaques.

completely suppressed. In the present experiment a ratio of 3 : 1 was used. Further evidence of decreased absorption of cholesterol in this experiment is provided by the absence of arcus senilis and the lower ratios of adrenal/carcass wt. in the group fed sitosterol.

The amount of atheroma in the aorta is considerably less in the rabbits fed cholesterol and sitosterol than in the group fed cholesterol only, the latter group showing the severe atheroma found in rabbits with high blood pressure. It seems reasonable to assume that the lesser degree of atheroma in the sitosterol-supplemented group is a consequence of the smaller amount of circulating cholesterol available for deposition in the aorta. The rabbits with the lowest levels of serum cholesterol in Group II are those showing the least amount of

atheroma. Conversely rabbit number 66, the rabbit with the greatest amount of atheroma in Group II, showed a moderately high serum cholesterol level for the greater part of the experiment. Numbers 34 and 70 although having a moderately high mean serum cholesterol level over the last 4 weeks showed only modest rises during the early part of the experiment, and this may account for their having less atheroma than number 66. The respective rôles of serum cholesterol level and blood pressure level are now under investigation.

The results of this experiment show clearly that even in the presence of a high blood pressure, sitosterol can adequately reduce the amount of atheroma produced by cholesterol feeding.

SUMMARY

Rabbits with high blood pressure were fed for 8 weeks on a diet containing either cholesterol alone or cholesterol + three times the amount of β-sitosterol.

Serum total cholesterol levels were significantly lower at all stages of the experiment in the group receiving β-sitosterol.

The amount of aortic atheroma was considerably lower in the group receiving β-sitosterol.

We are grateful to Dr. R. E. Shipley of Eli Lilly & Co., Indianapolis, Indiana, U.S.A. for the supply of β-sitosterol, and to Mr. A. Beasley and Miss Stella Hardie for much help during this experiment.

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THE EFFECT OF A BRIEF PERIOD OF HIGH BLOOD PRESSURE ON CHOLESTEROL-INDUCED ATHEROMA IN RABBITS

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In a previous investigation into the effect of high blood pressure on the severity of cholesterol-induced atheroma in rabbits (Bronte-Stewart and Heptinstall, 1954), it was noticed that 2 animals which underwent a spontaneous drop in pressure from their previous high levels had atheroma comparable in severity with those with a high pressure for the whole of the experiment. From this it appeared that a short period of high blood pressure might accentuate the amount of atheroma produced. In the present experiment the truth of this was tested, and it was found that in an 8-week feeding period, a high blood pressure for the first 2 or 3 weeks could increase the amount of aortic atheroma produced, although not to the same extent as in rabbits with a high pressure for the whole 8 weeks.

MATERIALS AND METHODS

Male Chinchilla and New Zealand White rabbits were divided into 3 groups. Group I consisted of 8 rabbits with high blood pressure produced by the removal of the right kidney and the application of a silver clip to the left renal artery 2 weeks later (Pickering and Prinzmetal, 1938). These were the rabbits referred to as Group I in the foregoing paper. The animals were fed diet 18 of Bruce and Parkes (1946) to which cholesterol was added (Heptinstall and Bronte-Stewart, 1954), the diet being adjusted so that each animal consumed between 3·5 and 4·2 g. cholesterol per week. Unlimited water was allowed and the total period of feeding was 8 weeks. Group II consisted of 7 rabbits in which a high blood pressure was produced in the same way and cholesterol feeding started as in Group I. After a period of between 14 and 21 days the silver clips were removed from the renal artery through the abdomen, with a resultant fall in blood pressure within 4 days to normal or near normal levels. Cholesterol feeding was maintained during this time and for the remainder of the 8-week period. Group III was a miscellaneous group of 7 rabbits acting as controls. Three were given a high blood pressure and the clip removed after 14–21 days as in Group II but differing in that no cholesterol was added to the diet during the whole of the period. After the blood pressure had fallen the rabbits were given the cholesterol-supplemented diet for 8 weeks. The remaining 4 were animals which failed to develop a high blood pressure or which showed an insignificant rise after removal of one kidney and the application of a silver clip to the other renal artery. These were fed the cholesterol diet for 8 weeks.

In all groups the animals were weighed and bled for serum total cholesterol estimations once weekly and blood pressures taken twice weekly as in the previous experiment (Heptinstall and Porter, 1957). After 8 weeks on the cholesterol diet the rabbits were killed and post-mortem examination carried out. The amount of atheroma in the aorta was recorded as described in the previous experiment and a " score " given to each rabbit. Adrenal, spleen, liver and carcass were weighed and paraffin and frozen sections made from the main organs.

RESULTS

Level of serum cholesterol

In most cases the serum total cholesterol reached a stable level after 4 weeks' feeding and for the remainder of the experiment remained at or near that level. Tables I and II show the mean levels over the last 4 weeks of feeding. There was no appreciable difference between the means in Groups I and II, while in Group III a higher level was maintained. An occasional rabbit showed a poorer response to feeding than the others and one such animal (number 49 in Group II) had a peak level of only 360 mg./100 ml. with a mean of 280 mg./100 ml.

TABLE I.—*Amount of Aortic Atheroma, Serum Total Cholesterol Level, Adrenal Wt./Carcass Wt. Ratio, Mean Blood Pressure and Mean Rise in Blood Pressure, for Groups I and III*

	Number of rabbit	Aortic atheroma score	Mean serum total cholesterol over last 4 wk. (mg./100 ml.)	Adrenal wt. × 100 / carcass wt.	Mean B.P. (mm. Hg)	Mean rise in B.P. (mm. Hg)
Group I	22	103	1450	0·070	114	42
	17	82	1110	0·042	120	46
	63	66	1590	0·074	105	30
	20	63	960	0·073	99	16
	31	41	1040	0·086	111	33
	48	40	990	0·051	100	28
	50	22	520	0·074	100	21
	15	15	1170	0·073	99	13
		Mean	1104±318	0·068±0·015		
Group III	11	11	760	0·069	81	0
	39	6	1390	0·150	91	12
	35	5	2730	0·075	78	0
	72	5	1350	0·101	80	13
	43	3	500	0·041	81	5
	62	2	1350	0·075	83	4
	37	1	2200	0·100	81	6
		Mean	1470±772	0·087±0·038		

Blood pressures

Rabbits in Group I maintained their high blood pressure (Table I) for the duration of the experiment with the exceptions of numbers 50 and 15 which showed a fall of 10–15 mm. Hg during the final 2 weeks. In spite of this their mean pressures for the duration of the experiment were 100 and 99 mm. Hg respectively.

Rabbits in Group II were allowed to have a high blood pressure for 14–21 days of cholesterol feeding, followed by removal of the clip on the left renal artery. This resulted in a fall in the blood pressure to normal or near normal levels within 4 days. The one exception, number 49, showed a fall of 17 mm. Hg but remained with a pressure 31 mm. above its pre-experiment level for the remainder of the feeding period. Table II shows the blood pressures at various stages of the experiment.

No rabbit in Group III (Table I) with the exception of number 39 exceeded a mean pressure of 83 mm. Hg during the experiment.

TABLE II.—*Amount of Aortic Atheroma, Serum Total Cholesterol Level, Adrenal Wt./Carcass Wt. Ratio, Levels of Blood Pressure at Different Periods for Group II*

Number of rabbit	Aortic atheroma score	Mean serum total cholesterol over last 4 wk. (mg./100 ml.)	Adrenal wt. × 100 / carcass wt.	Period of high B.P. (wk.) and level of B.P. (mm. Hg) Time	B.P.	Period of low B.P. after clip removal (wk.) and level of B.P. (mm. Hg) Time	B.P.	Mean level of B.P. for whole experiment (mm.Hg)	Mean pre-operative B.P. (mm.Hg)
26	45	1230	0·052	2	111	6	88	94	85
8	21	1310	0·078	2	99	6	78	84	80
53	15	930	0·040	3	100	5	83	88	78
44	12	910	0·080	3	100	5	80	89	79
49	8	280	0·060	2	120	6	103	110	72
59	8	1900	0·060	2	101	6	81	87	72
13	2	1340	0·083	3	97	5	84	91	75
	Mean	1130±357	0·065±0·018						

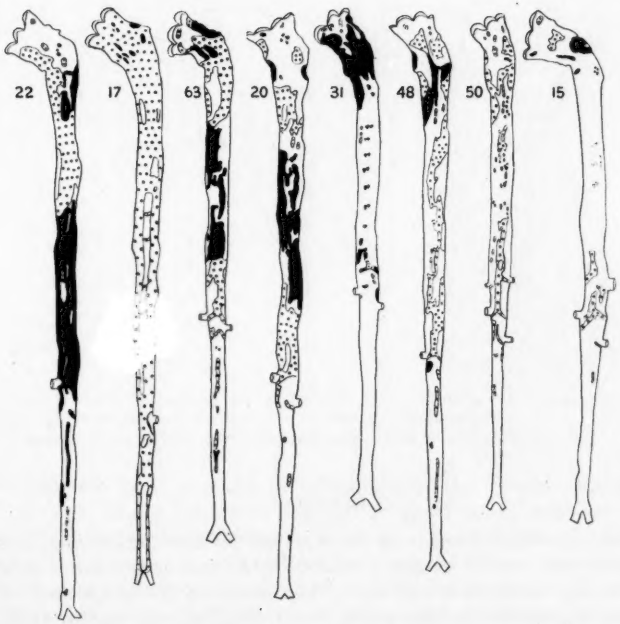

FIG. 1.—Amount of aortic atheroma in Group I (3/5 natural size). Dark areas represent prominently raised plaques and stippled areas slightly elevated plaques in this and in Fig. 2 and 3.

Amount of aortic atheroma

The amount of atheroma in the aorta in all three groups is adequately shown in Tables I and II and Fig. 1, 2 and 3. It will be seen that the amount in Group II is less than in Group I but greater than in Group III when the groups are considered as a whole.

Visceral changes

The usual visceral changes found in cholesterol-fed rabbits were present to a comparable degree in all 3 groups. Only one feature need be stressed and this is the adrenal wt./carcass wt. ratios. This has been shown to be a crude index of cholesterol absorption (Heptinstall and Bronte-Stewart, 1954), and in the present experiment is seen to be equal in Groups I and II but higher in Group III.

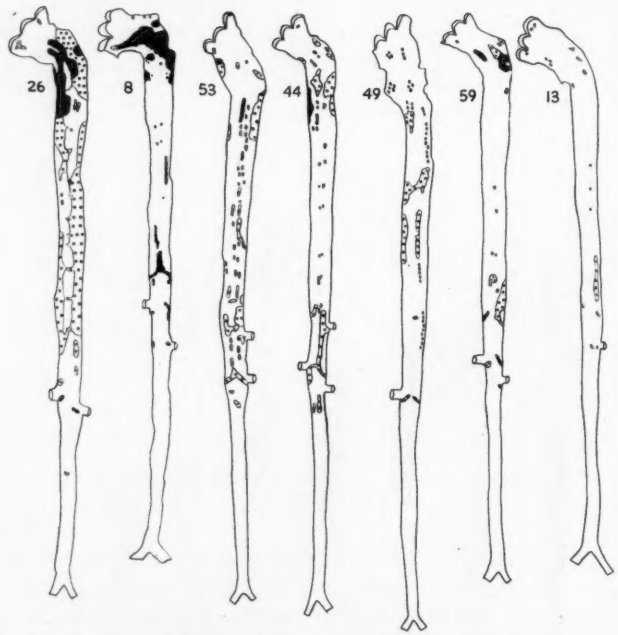

FIG. 2.—Amount of aortic atheroma in Group II (3/5 natural size).

DISCUSSION

To account for the difference in the amount of aortic atheroma in the 3 groups, certain factors can at once be eliminated. The duration and amount of cholesterol feeding were the same in all groups. The amount of cholesterol absorbed and available for deposition in the aorta is reflected in the serum total cholesterol levels and adrenal wt./carcass wt. ratios. Both of these are identical in Groups I and II but somewhat higher in Group III, the control group, a situation which hardly favours Groups I and II. All the animals used were of the same sex and

the proportions of Chinchilla and New Zealand White rabbits were the same in all groups. It is therefore evident that the level of the blood pressure is of the greatest importance in determining the amount of atheroma produced. A raised pressure during the whole of the feeding period produces considerably more aortic atheroma than a normal pressure, confirming the results of a previous experiment (Bronte-Stewart and Heptinstall, 1954). It will also be seen that the 2 rabbits in Group I which showed a terminal fall in pressure (numbers 50 and 15)

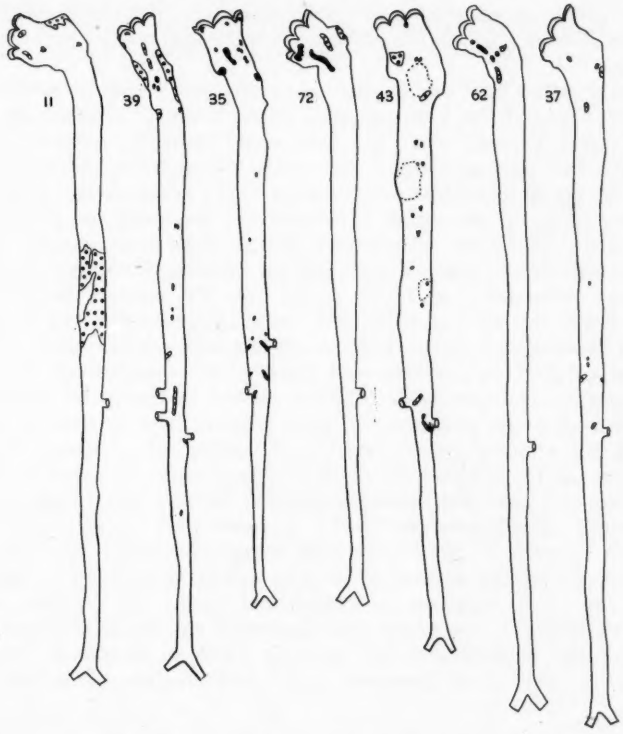

FIG. 3.—Amount of aortic atheroma in Group III (3/5 natural size). Areas enclosed by dotted line in rabbit 43 represent areas of spontaneous medial disease.

are the two with the least amount of atheroma in the group. Rabbits with a brief period of high blood pressure, Group II, show more atheroma than the control Group III but less than Group I. This, in certain measure, confirms what had been suspected in a previous experiment. Briefly it had been found that in a 10-week feeding experiment, 2 rabbits which had maintained blood pressures of 128 and 119 mm. Hg respectively for 5 weeks suddenly showed a drop in pressure. During the last 5 weeks of the experiment the blood pressures of both fluctuated between 88 and 98 mm. Hg. At post-mortem examination both were found to have aortic atheroma comparable with rabbits maintaining a high pressure for

the whole 10-week period. It was therefore decided to repeat this with an even briefer period of high blood pressure. The briefer period was partly conditioned by the observation of Blacket and Sellers (1951) that the fall in pressure is slower the longer the clip is in position before removal. In general in the present experiment the clip was removed 4–5 weeks after application, 2 weeks being allowed to make sure that the pressure was rising before cholesterol feeding was started, and 2–3 weeks on cholesterol being allowed before clip removal. The amount of aortic atheroma found in the rabbits of Group II was not so great as in the 2 rabbits in which the original observation was made, but the present experiment was 2 weeks shorter and the initial high pressure was neither so high nor so long maintained.

The reasons why a brief rise in pressure accentuates atheroma are not altogether clear. If the effect of the high pressure were merely to increase the passage of cholesterol into the vessel wall, then one would expect to see evidence of aortic atheroma at a time corresponding to the end of the period of high pressure. Examination of the aorta of rabbits with a high blood pressure fed cholesterol for 3 weeks shows no gross deposition of cholesterol and only insignificant amounts microscopically. However, it must be stated that examination of the aorta from the endothelial surface as described by Lautsch, McMillan and Duff (1953) has not been attempted, and this might provide information not ordinarily apparent. Local injury might provide an explanation for the increased lipid deposition. Examination of the aorta of rabbits on a normal diet with high blood pressures of a degree comparable with those in the experiment has revealed no obvious structural changes. Also 3 of the rabbits in Group III (numbers 11, 35 and 43) had high blood pressures for short periods prior to their pressures being reduced by clip removal before cholesterol feeding was started. These rabbits showed scores of 11, 5 and 3 which might have been expected to be higher if aortic damage had occurred during the period of high blood pressure. However it is conceivable that injury produced in a vessel wall during cholesterol feeding may be more potent in producing lipid localisation than an injury sustained before cholesterol feeding is started. It may well be as Duff (1954) has speculated, that when the whole organism is flooded with lipids, the smallest structural or functional aberration of the vessel wall may serve to localise the lipid.

Whatever the explanation, the findings serve to emphasise the important rôle played by high blood pressure in the accentuation of cholesterol-induced atheroma.

SUMMARY

Rabbits were divided into 3 groups all of which were fed cholesterol 0·5 g. per day for 8 weeks.

The first group consisted of animals which had a high blood pressure for the whole of the experiment. The second group had a high blood pressure for the first 2 or 3 weeks and a normal or near normal pressure for the remainder of the 8 weeks. The third group had normal or only slightly elevated pressures for the whole duration of the experiment. Aortic atheroma in the second group was greater than in the third but not so great as in the first.

We are grateful to Mr. A. Beasley and Miss Stella Hardie for their assistance during this experiment.

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FLUID TRANSFER FROM THE PERITONEAL TO THE PLEURAL CAVITIES IN RODENTS

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INFORMATION about the transfer of fluid between the peritoneal and pleural cavities is scanty, although most authorities accept the view that there is a direct channel of communication across the diaphragm. The existence of such a channel is commonly postulated to explain the concurrence of ascites and hydrothorax in Meigs' syndrome and in many cases of hepatic cirrhosis. In theory, it might take the form of pores in the serous membranes (the evidence is reviewed by Drinker and Yoffey (1941)), or there might be a true diaphragmatic hiatus as is sometimes obvious in congenital diaphragmatic malformations (Sweet, 1950; Deacon, 1954), or the diaphragmatic lymphatics might provide the channel of transport (Meigs, Armstrong and Hamilton, 1943; Meigs, 1954; Cowan, Cron, Burgess and Karioris, 1954).

The present experiments were designed to determine whether artificially induced ascites causes hydrothorax in rodents and to study the means whereby such fluid transfer may occur.

METHODS

Rabbits weighing 2–3 kg. and rats weighing 250–350 g. were given mammalian Ringer solution (in some experiments mixed with methylene blue, India ink or colloidal graphite) intraperitoneally, either by continuous drip over a 4–5 hr. period or by rapid infusion completed in a few minutes. The drips were set up or the infusions administered with sterile precautions under ether anaesthesia, preceded in the rabbits by intravenous nembutal induction. The volume of fluid injected was 350–500 ml. in the rabbits and 35–60 ml. in the rats, and the duration of the experiments was usually 5–7 hr. though an occasional animal was observed for a shorter or longer period (2–24 hr.). At the end of the observation period the animals were killed, their serous cavities examined immediately, and any fluid present was measured. The diaphragms of the animals which had the India ink or colloidal graphite injections were fixed in 10 per cent formol-acetate for histological examination. To assess the influence of simple hydration in the preceding experiments, two groups of control animals were given subcutaneous fluid by continuous drip in like or larger volume. Urine output was measured in all the drip experiments. These animals required partial immobilization in their cages to prevent kinking of the drip tubes and this was accomplished by the application of plaster-of-Paris jackets which were loosely tethered to the cage-sides.

RESULTS

As shown in the accompanying Table, hydrothorax developed in all the rabbits and rats in which artificial ascites had been induced by the intraperitoneal injections, while no serous effusions occurred in those receiving subcutaneous fluid. The animals which received intraperitoneal injections showed considerable variation in the rate of absorption of fluid from the peritoneal cavity, but whether administered by slow drip or by rapid infusion, 25–80 per cent of the fluid had

disappeared in 5–7 hr. Fluid (up to 10 per cent of the original volume injected) was always found in the pleural sacs; there was no sign of any special preponderance in either pleural cavity which could not be accounted for by the posture of the animal during the experiment. The results suggest that less pleural fluid appears when the injection is made by single rapid infusion than by slow drip. The difference, which was more marked in the rabbits than in the rats, is probably a real one and perhaps due to the relatively greater restriction of diaphragmatic movement that arises from a single massive intraperitoneal infusion. On the other hand, the plaster-of-Paris jackets employed in the drip experiments probably had no influence on fluid absorption from the peritoneal cavity or on its transfer to the pleural cavity. This was apparent from control experiments in which rapid intraperitoneal infusions were given to 6 rabbits and 6 rats immobilized in plaster in the same way as before; the results were not materially different from the rapid infusion experiments without plaster jackets. A series of 7 intraperitoneal drip experiments, 5 of which were in rabbits, maintained for shorter or longer periods than the 5–7 hr. of the main experimental groups, is not included in the Table. These gave confirmatory results: there was proportionately more pleural fluid and less ascites as the duration of the experiment increased, *e.g.*, the measurements in a 12-hr. experiment in a rabbit were 200 ml. peritoneal and 65 ml. pleural fluid.

TABLE.—*Effects of Injecting Mammalian Ringer Solution Intraperitoneally or Subcutaneously in Rats and Rabbits*

Animal	No.	Procedure	Duration of expt. (hr.)	Volume of fluid (mean and range) ml.			
				Injected	Peritoneal	Pleural	Urine
Rabbits	6	Slow intraperitoneal drip	5	450 (350–500)	240 (150–400)	32 (7–52)	69 (20–150)
„	6	Rapid intraperitoneal infusion	7	400	290 (250–320)	9 (4–18)	—
„	6	Slow subcutaneous drip	6–7	550 (380–1000)	0	0	118 (85–150)
Rats	6	Slow intraperitoneal drip	5	55 (35–60)	28 (10–43)	3 (1·5–6)	4 (2·5–6)
„	10	Rapid intraperitoneal infusion	5–7	60	25 (15–35)	2 (1–4)	—
„	6	Slow subcutaneous drip	7	65 (50–85)	0	0·1 (0–0·2)	10 (5–12)

The hydrothorax which developed in the present experiments might, in theory, have been the result of simple overhydration of the animals. This explanation is, however, a most unlikely one since rats and rabbits hydrated to the same or greater degree by subcutaneous drip (Table) failed to develop effusions (0·2 ml. in the pleural cavity of one rat is no more than may be found normally). It is noteworthy that the urine output of these animals was greater than in the intraperitoneal drip experiments, indicating that absorption of fluid from the subcutaneous depots was faster than from the peritoneal cavity.

Dye Experiments

Ringer solution containing 0·1 per cent methylene blue was administered to 3 rabbits and 3 rats by rapid intraperitoneal infusion in the same volumes as

before. The addition of the dye had no demonstrable effect on the distribution of fluid : the rabbits had a mean volume of 260 ml. peritoneal fluid and 11 ml. pleural fluid ; the rats had 26 ml. and 3 ml. The fluid in the chest was, however, less deeply stained than in the abdomen, indicating that the fluid channel between the two cavities was not a simple opening or collection of openings.

This observation was elaborated in 6 similar experiments in rabbits and 6 in rats using injection solutions of Ringer containing 1 per cent India ink. The mean values obtained in the rabbits were 290 ml. peritoneal fluid and 7 ml. pleural fluid, and in the rats, 41 ml. and 3 ml. Again, the dye was present in lower concentration in the chest and in one rat the pleural fluid looked quite unstained. No special precaution, beyond ordinary care, was taken in opening the pleural cavities in these animals and it is possible that some of the relatively lightly staining fluid here was contaminated by India ink from incised pleural lymphatics. Subsequent histological examination demonstrated that India ink was in fact present in these lymphatics.

To obtain further information about the lymphatic absorption of peritoneal fluid a further 7 rats were given a 10 per cent suspension of colloidal graphite in 60 ml. Ringer by single injection intraperitoneally. (The India ink was found to be too toxic for parenteral administration in such high concentration.) These rats, killed after 7 hr., had peritoneal fluid ranging in volume from 33–40 ml. (mean 37 ml.), while the pleural fluid volume was only 0·2–0·4 ml. (mean 0·2 ml.) : *i.e.*, compared with the measurements in rats which had infusions of pure Ringer, the volume of pleural fluid was significantly reduced while the volume of peritoneal fluid was increased. The reduction of fluid transfer from the peritoneal cavity might reasonably be explained as an effect of lymphatic obstruction by graphite ; this view receives support from histological examination of the diaphragm (Fig. 1–4) which showed intense pigmentation of the peritoneal lymphatics while those in the substance of the diaphragm and on the pleural surface contained fewer carbon particles.

DISCUSSION

The present observations supply evidence that there is in rabbits and rats a more direct means of fluid transfer between the peritoneal and pleural cavities than is provided by the general fluid exchanges of the circulatory system. The channel appears to be incompletely permeable rather than an open communication

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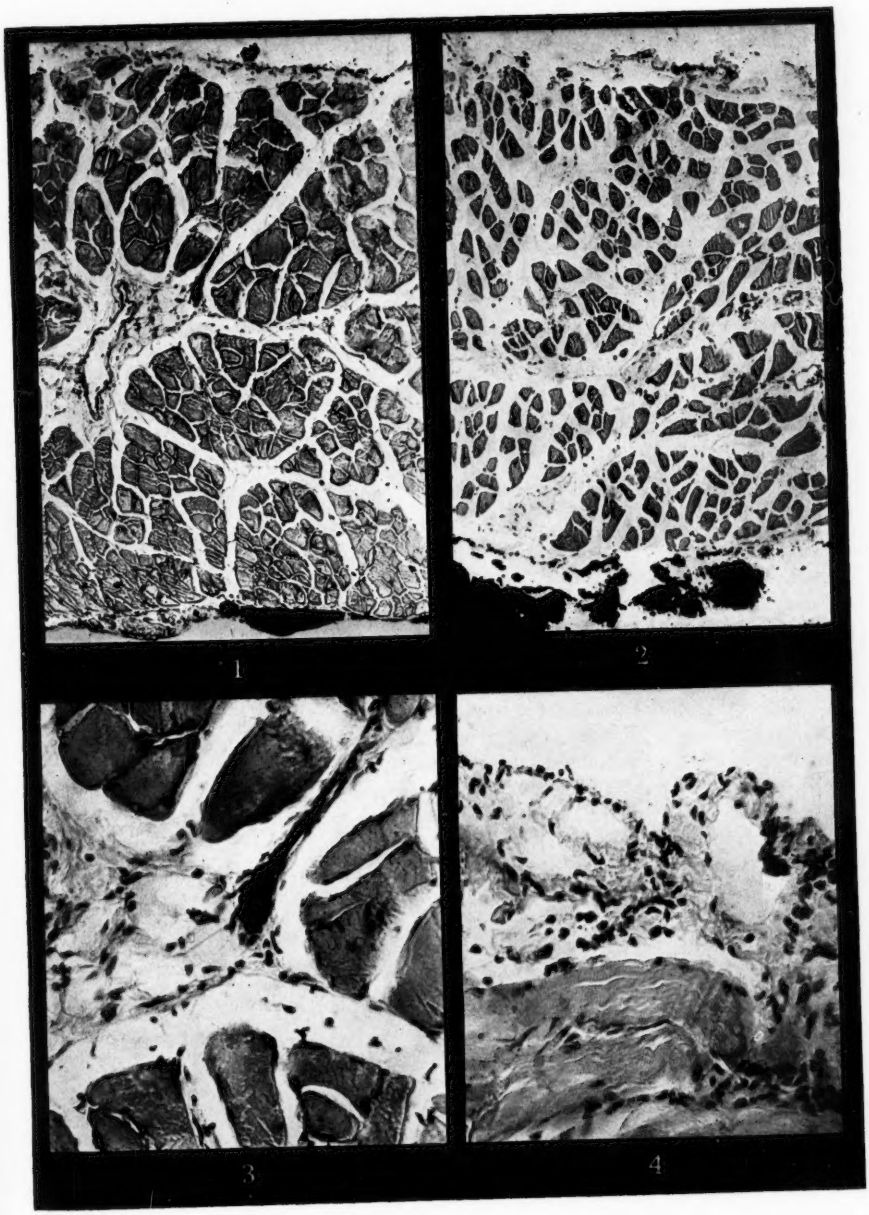
EXPLANATION OF PLATE

FIG. 1.—Rat diaphragm after the intraperitoneal injection of 10 per cent graphite. There is abundant pigment in the subdiaphragmatic lymphatics ; a smaller amount of pigment is present in the paravascular lymphatic in the substance of the diaphragm and in the lymphatics on the pleural surface. The largest mass of pigment on the pleural surface is lying free. (H. and E. ×94.)

FIG. 2.—Rat diaphragm after the intraperitoneal injection of 10 per cent graphite showing abundant pigment lying free below the diaphragm and in the subdiaphragmatic lymphatics. A lymphatic containing a small amount of pigment is visible on the pleural surface. (H. and E. × 94.)

FIG. 3.—High power view of Fig. 1 showing the pigment-filled paravascular lymphatic in the substance of the diaphragm. (× 280.)

FIG. 4.—High power view of lymphatics on the upper surface of diaphragm ; clumps of graphite pigment are visible in two of the lymphatics. (H. and E. × 280.)



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since colloidal particles traversed the diaphragm less readily than the crystalloid solution. When the local lymphatics were blocked by a heavy concentration of graphite, fluid transfer from the abdomen to the chest was diminished, suggesting that the diaphragmatic lymphatic system is the important link between the serous cavities. The alternative explanation, that the cavities communicate by way of microscopic pores in the diaphragm, is an unlikely one for several reasons. If there were open pores in the diaphragm, it should be possible to transfer air as well as fluid from the abdomen to the chest or in the opposite direction. Direct experiment in the present study showed that this could not be done in rats or rabbits. It is also everyday clinical experience that pneumothorax does not cause pneumoperitoneum and vice versa, which is in accord with the experiments quoted by Meigs *et al.*, on cases of Meigs' syndrome, showing that air introduced into the chest or abdomen failed to traverse the diaphragm. There is, moreover, no satisfactory morphological evidence that diaphragmatic pores exist, and in the histological preparations of the diaphragm from the present dye experiments, extra-lymphatic pigment was not seen. If it may be concluded that the lymphatics are the main fluid channel across the diaphragm, their unusual rôle in discharging fluid into the chest instead of absorbing it is likely to be an accidental one, due perhaps to leakage from over-filling. Respiratory movements doubtless have some influence here both in increasing the rate of lymphatic absorption below the diaphragm and in exposing lymphatic channels in the chest to transient intermittent reductions in external pressure.

It is common experience that ascites, in experimental animals and in man, is frequently associated with hydrothorax. Marshall (1949) states that all his autopsies of cases of ascites showed some pleural effusion, and although this is not everyone's experience, it emphasizes the frequency with which the two conditions are associated. One of the questions which prompted the present investigation was : can hydrothorax, in the presence of ascites, be ascribed solely to the local permeation of fluid or must some general cause of the serous effusions be postulated? The results indicate that substantial hydrothorax can rapidly develop in experimental animals from the transdiaphragmatic passage of ascitic fluid. The possibility that human ascites behaves similarly is supported by the tracer experiments of Cowan *et al.* (1954) in a clinical case of ascites and hydrothorax in which they demonstrated a more rapid transport of fluid across the diaphragm than could be accounted for by general circulatory fluid exchanges.

SUMMARY

The induction of artificial ascites in rabbits and rats by the intraperitoneal injection of mammalian Ringer solution led to the rapid development of substantial pleural effusions. This was not simply an effect of over-hydrating the animals since serous effusions did not develop when the solution was administered in like or greater volume subcutaneously.

When the fluid injected into the peritoneal cavity was mixed with methylene blue or India ink it was found that the colloidal particles passed less readily into the chest than the crystalloid solution. Moreover, when a more concentrated colloidal suspension was used (10 per cent graphite) the transfer of fluid from the abdomen was reduced, probably because the lymphatics were obstructed by the pigment. Histological examination confirmed that the subdiaphragmatic lym-

phatics were in fact filled with graphite which was also readily demonstrable, although in smaller amount, in the lymphatics within and above the diaphragm.

The findings indicate that there is a direct transdiaphragmatic fluid pathway between the peritoneal and pleural cavities which is probably provided by the local lymphatics.

I wish to thank Professor J. S. Young for his interest in this paper.

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THE EFFECT OF THE CONTINUOUS RE-INFUSION OF LYMPH AND LYMPHOCYTES ON THE OUTPUT OF LYMPHOCYTES FROM THE THORACIC DUCT OF UNANAESTHETIZED RATS

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THE output of lymphocytes from the thoracic duct of dogs (Yoffey, 1936) and rabbits and cats (Sanders, Florey and Barnes, 1940) is sufficient to replace all the lymphocytes in the blood several times daily. Thoracic duct cannulation gives a minimum figure for this daily replacement since lymphocytes enter the blood by other routes. Hughes, May and Widdicombe (1956) estimated that the combined output from the thoracic, cervical, subclavian and right lymphatic ducts of the rabbit would replace the lymphocytes in the blood about eleven times daily.

This idea of a rapid turnover of the blood lymphocytes is based on cell counts on part of the first day's flow of lymph; it assumes that the high figures for the lymphocyte output obtained during the first day represent the true continuous output in the intact animal.

Bollman, Cain and Grindlay (1948) made a notable advance when they described a method for the continuous collection of thoracic duct lymph from unanaesthetized rats for periods up to 10 days. Using this method, Mann and Higgins (1950) confirmed that the initial output of lymphocytes was very high but they found that when the drainage of lymph was prolonged the daily output of cells progressively fell; by the 4th day after cannulation it had regularly fallen to about 30 per cent of the initial value. This fall in output was independent of the rate of lymph flow and was not due to the operative trauma or to the loss in body weight that occurred. Mann and Higgins suggested that the fall in lymphocyte output might be due to the loss from the animal of a small number of dividing cells that were essential for maintaining lymphocyte production.

In the present work an apparatus is described for the continuous intravenous re-infusion of lymph and lymphocytes into unanaesthetized rats with thoracic duct fistulae. The apparatus allowed measurements of the lymphocyte output from the thoracic duct to be made, and it reproduced the situation found in the intact animal by returning all the lymph to the venous blood. Assuming that the first day's output of lymphocytes from a thoracic duct fistula represents the true output in the intact animal, then the continuous intravenous re-infusion of lymph and living lymphocytes would be expected to maintain the output from the fistula at its level on the first day. Thus, the first aim in this investigation was to discover whether the fall in lymphocyte output described by Mann and Higgins could be prevented by returning to the animal all its lymph and lymphocytes.

The second aim was to explain the fall in lymphocyte output that occurs when lymph and lymphocytes are lost from the animal. For this purpose a comparison was made between the output of cells from the thoracic duct of rats in which lymph and living lymphocytes were re-infused and the output from rats receiving

continuous re-infusions of either cell-free lymph or lymph and killed lymphocytes. It was hoped that this would provide a test of Mann and Higgins' suggestion that the fall in output might be due to the loss from the animal of cells essential for maintaining lymphocyte production.

In these experiments, the viability of the re-infused lymphocytes was tested by observing their behaviour *in vitro*. Motility of the cells was taken as the criterion of viability. While performing these tests some additional observations were made on the properties of the cells in rat thoracic duct lymph.

METHODS

Male albino rats weighing 145–200 g. were used. The thoracic duct was cannulated with polythene tubing (internal diameter 0·5 mm.) just below the diaphragm by the method of Bollman *et al.* (1948). This procedure drained lymph from the liver and intestine as well as from the muscles and skin below the diaphragm but injections of dye failed to show any contribution from the lymphatics of the head, neck and front limbs. After operation the rats were placed in a restraining cage (Bollman, 1948) to which a hopper for food and a drinking bottle were attached (Fig. 2). The rats were fed on commercial rat cake and given a solution of either 10 per cent glucose or of 0·9 per cent sodium chloride to drink. The latter was given to increase the flow of lymph if this showed any tendency to clot in the cannula. The restraining cage was covered with a cloth and care was taken not to disturb the rats by noise. The animals were lively and well when removed from the cage 4–14 days later.

Lymph was collected at room temperature into sterile graduated centrifuge tubes which contained 0·5 ml. of Hanks' solution (Hanks, 1948) for each 5–10 ml. of lymph. The Hanks' solution used in all experiments contained 200 μg. each of penicillin and streptomycin per ml.; for the collection of lymph it contained, in addition, 25 units of heparin per ml. Measurements of the lymph flow and cell counts were made on successive 3-hr. samples collected during a 12-hr. period each day.

The morphology of the cells in thoracic duct lymph was studied in stained films. Rous (1908) pointed out the difficulty of making satisfactory preparations of lymphocytes by smearing thoracic duct lymph. In the present experiments, films were made by first centrifuging the lymph and re-suspending the pellet of cells in a very small volume of rabbit

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EXPLANATION OF PLATES

FIG. 2.—Apparatus for the continuous re-infusion of lymph containing lymphocytes killed by ultra-violet light. The rat cage, with drinking bottle and hopper attached, stands in front of the tall oblong box which houses the ultra-violet lamp. The water-cooled quartz vessel, through which the lymph in the polythene coil is irradiated, is encircled by a metal ring on the front of the housing. The roller pump with the reservoir and a collecting tube (both containing lymph) are on the right of the cage.

FIG. 3 and 4.—Cultures of rat thoracic duct lymphocytes incubated at 37° for 7 hr. The lymph from which the cells in Fig. 3 were obtained, was exposed to ultra-violet light for 1 min. before the preparation of the culture; virtually all the cells are dead. The cells in Fig. 4, the non-irradiated control, are all alive. Phase contrast, × 525.

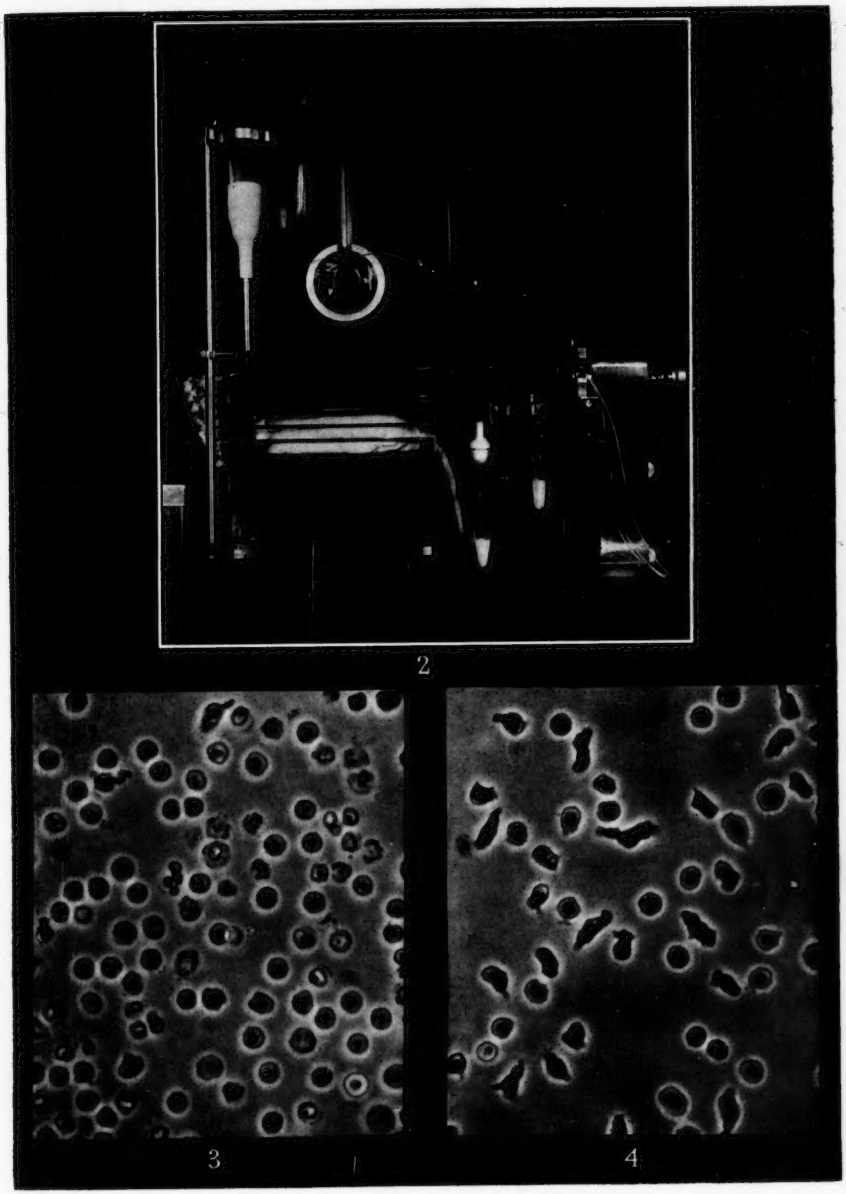
FIG. 5–8.—Photographs at 1 min. intervals of the same field in a culture of rat thoracic duct lymphocytes incubated at 37° for 3½ hr. The shapes assumed by the two large lymphocytes in the centre of the field are very similar to those of the small lymphocytes in the same field and in Fig. 4. Phase contrast, × 525.

FIG. 9–11.—Stages in the division of large lymphocytes in films of rat thoracic duct lymph. Wright's stain, × 900.

FIG. 12–15.—Division of a large lymphocyte. Phase contrast, × 525.

FIG. 12.—Culture of rat thoracic duct lymphocytes incubated at 37° for 1 hr. The large lymphocyte, slightly to the right of centre, is about to divide.

FIG. 13–15.—The same field as Fig. 12 photographed 30, 33 and 110 min. later. In Fig. 15 the two daughter cells, roughly one above the other in the centre of the field, have separated. They are larger than the small lymphocytes.



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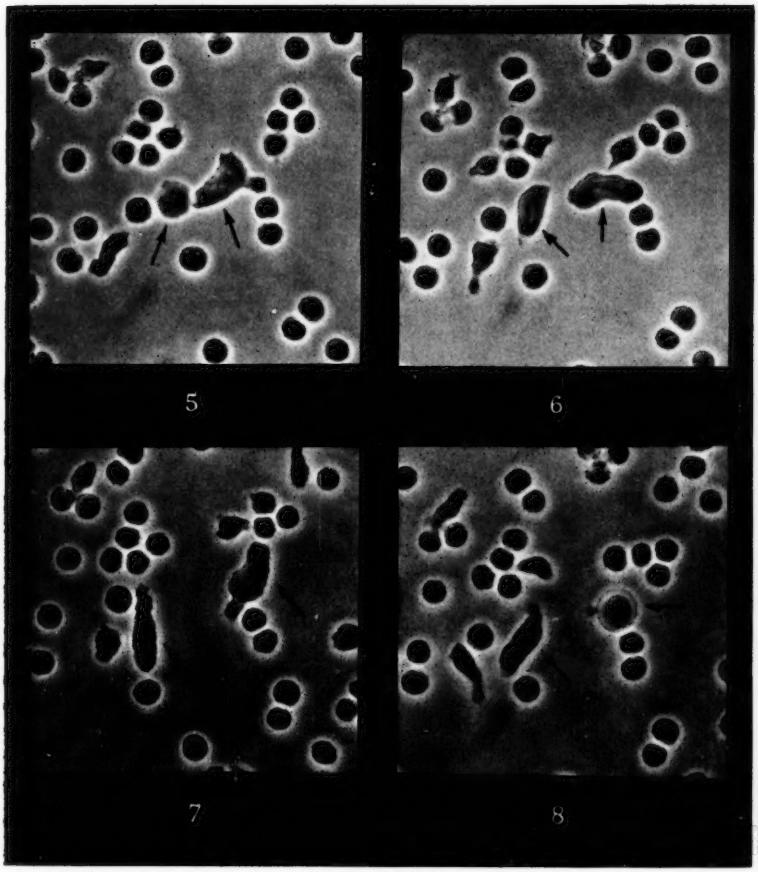
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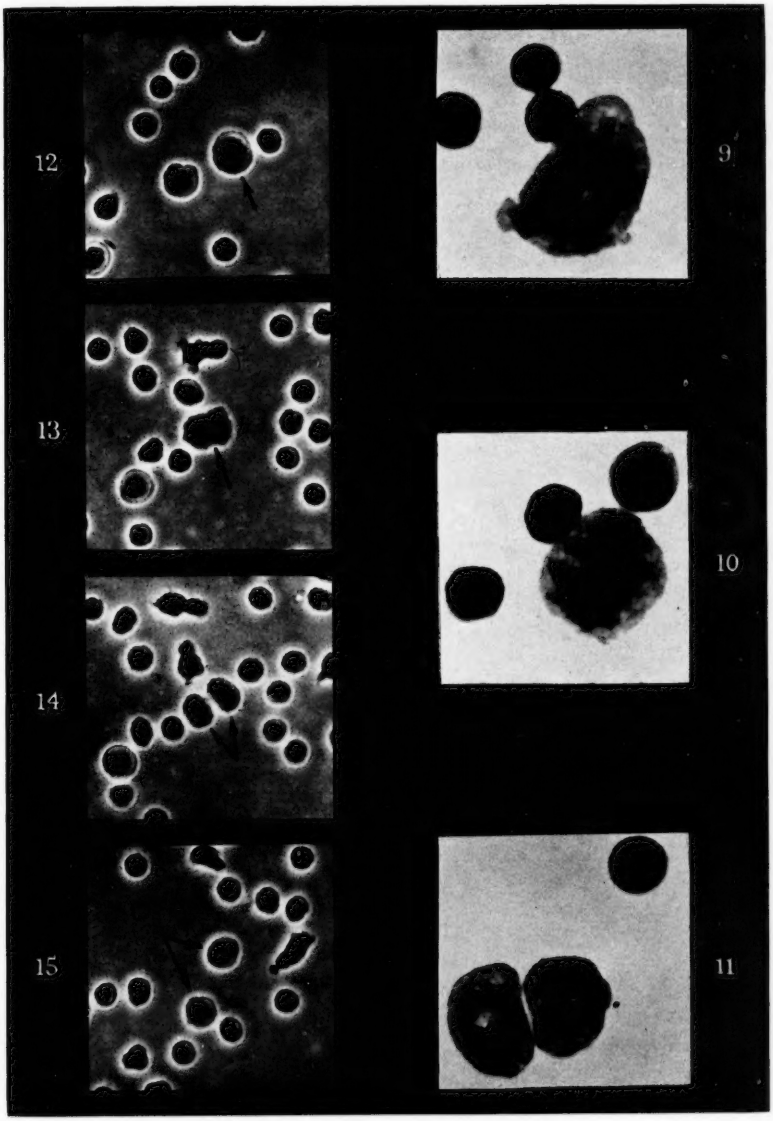

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serum. Smears of the thick suspension of cells were then made in the usual way, dried in air and stained with Wright's stain.

Blood for leucocyte counts and haemoglobin estimations was taken from the tail of unanaesthetized rats after they had been warmed for some minutes on an electric heating pad. Haematocrit values were obtained from the heart blood at the end of the experiment.

Intravenous re-infusion of lymph and living lymphocytes

The apparatus (Fig. 1 and 2) was designed to re-infuse all the lymph intravenously at approximately the rate at which it had flowed from the thoracic duct. Measures were taken to prevent clotting of the lymph and to ensure that the lymphocytes were alive when they entered the rat.

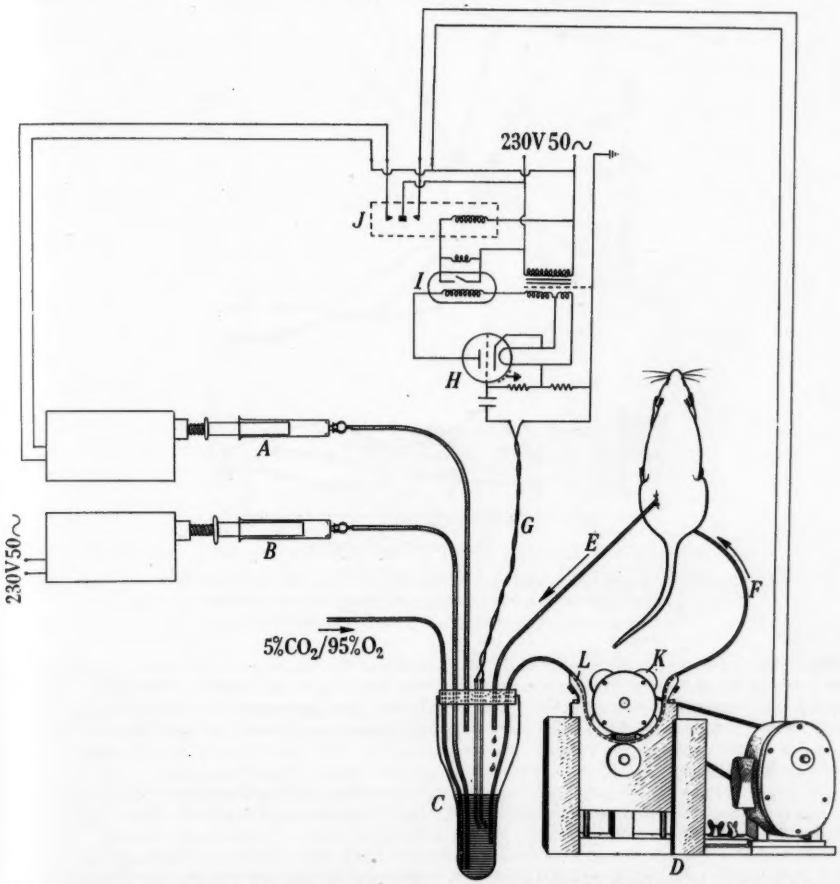

FIG. 1.—Apparatus for the continuous intravenous re-infusion of lymph and lymphocytes into rats with thoracic duct fistulae. A and B: electrically driven syringes; C: siliconed glass reservoir; D: roller pump; E: cannula from thoracic duct; F: cannula returning lymph to femoral vein; G: connection between relay circuit and control electrodes; H: thyratron, T41; I: "Sunvic" H.V.S.; J: change-over relay; K: small wheels which compress polyvinyl tubing lying in semi-circular groove L. For further explanation see text.

The lymph collected during the day for cell counts was pipetted into a siliconed glass reservoir (C in Fig. 1) from which it was pumped continuously into the femoral vein of the rat by a roller pump (D). The pump had an adjustable delivery of between 0·5 and 4·0 ml. per hr. The pumping circuit from the reservoir to the rat consisted entirely of plastic tubing. Polyvinyl tubing from the reservoir was joined to a 2·5 cm. length of rigid nylon tube (internal diameter 0·5 mm.) which was clamped at the inlet of the pump. Four small wheels (K) rolled in a semi-circular groove (L) in the main block of the pump. A length of polyvinyl tubing (internal diameter 0·5 mm.) lying in this groove was attached at one end of the nylon

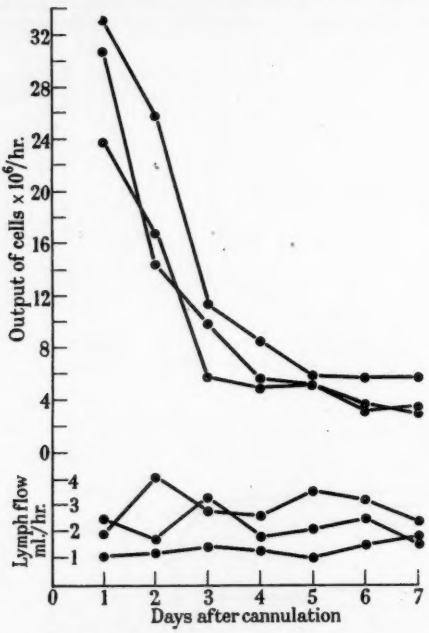

FIG. 16.—Output of lymphocytes and rate of lymph flow from the thoracic duct in 3 rats. Mean values per hr. for each successive 24 hr. after cannulation.

tube at the inlet and rode free at the outlet to allow for changes in its length as the wheels compressed it. A thin phosphor-bronze strip was fitted into the length of the groove to hold the tubing permanently in position. The metal strip also protected the tubing from excessive wear by the wheels. At the outlet, the polyvinyl tube was joined by a polythene adaptor to a length of fine polythene tubing (internal diameter 0·25 mm.; F in Fig. 1) which passed directly into the femoral vein of the rat.

A 5 per cent CO_2/95 per cent O_2 gas mixture was bubbled continuously through the lymph and Hanks' solution in the reservoir. This maintained the pH at about 7·4 and kept the lymphocytes in suspension.

During the day the pump could be adjusted to keep the volume of lymph in the reservoir at a minimum. At night, when no samples were taken for cell counts, the lymph dripped directly into the reservoir. If the pumping rate had exceeded the rate of lymph flow the reservoir would have emptied and air would have been pumped into the rat. In order to avoid this, a thyratron-activated relay automatically switched off the pump when the electrical circuit across the electrodes (G) was broken by the fall in the level of the fluid in the reservoir. At the same time, the relay switched on an electrically-driven syringe (A) which delivered into the reservoir a volume of Hanks' solution containing 1 unit of heparin per ml. When

fluid covered the electrodes again the syringe was automatically switched off and the pump re-started.

At night, to prevent clotting of lymph, Hanks' solution containing 25 units of heparin per ml. was pumped continuously into the reservoir at a rate of 0·3 ml. per hr. by a second electrically-driven syringe (B). The reservoir and pumping circuit were washed out morning and evening to remove any small clots or débris. This was particularly necessary on the first day when traces of blood were sometimes present in the lymph.

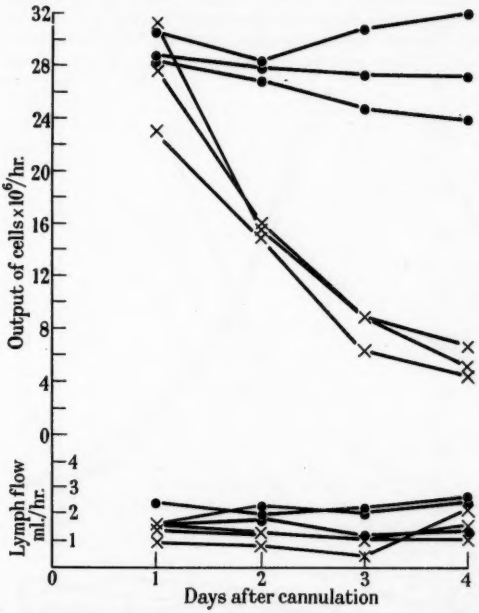

FIG. 17.—Output of lymphocytes and rate of lymph flow from the thoracic duct in 6 rats. Mean values per hr. for each successive 24 hr. after cannulation.

●———● rats with continuous intravenous re-infusion of lymph and living lymphocytes. ×——× rats with no re-infusion.

Viability of lymphocytes

Tests were made to ensure that the re-infused lymphocytes were alive when they entered the rat. It was decided that the only satisfactory criterion of viability was motility of the cells *in vitro*.

Cultures were prepared with lymph which had been collected from the outlet of the pump and with lymph collected into heparinized Hanks' solution directly from the thoracic duct of the same rat. A comparison of the behaviour *in vitro* of the lymphocytes in these two series of cultures was made to determine if the cells had been damaged by their passage through the pump.

The culture medium for the lymphocytes consisted of equal parts of Hanks' solution and inactivated (56° for 20 min.) Seitz-filtered serum. Old serum from rabbits was usually employed but in some experiments the medium contained the rat's own (autologous) serum which had been obtained by cardiac puncture 1–2 weeks before thoracic duct cannulation.

The cultures were optically better if the lymph contained very little fat. This was ensured by fasting the rats for a few hours and giving them a solution of 0·9 per cent sodium chloride to drink. The heparinized lymph from the collecting tubes or from the pump outlet was

added to the culture medium to give a final cell concentration of 1000–2000 cells per cu. mm. The culture medium containing the cells was gassed with 5 per cent CO_2/95 per cent O_2 to bring the pH to 7·4. It was then introduced into the perspex coverslip chambers devised by Mackaness (1952). Lymphocytes do not adhere to glass and they were viewed through the lower coverslip of the chamber with an inverted phase-contrast microscope. The observations were carried out in an incubator room at 37°.

The living cells in the cultures were recognized by their amoeboid movements. The character of these movements conformed to the descriptions given by Lewis (1931, 1933),

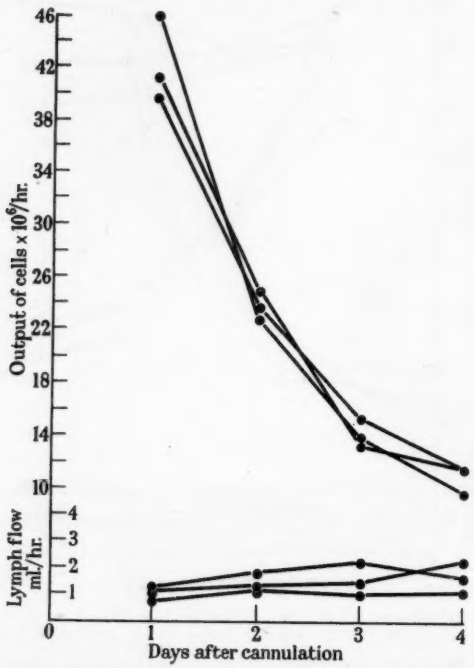

FIG. 18.—Output of lymphocytes and rate of lymph flow from the thoracic duct in 3 rats. Mean values per hr. for each successive 24 hr. after cannulation. The rats received a continuous intravenous re-infusion of cell-free lymph.

Clark, Clark and Rex (1936) and de Bruyn (1945) and no further account of them will be given here. Dead lymphocytes were recognized by the permanent rounded-up form of the cell. At a later stage the dead cells developed a prominent nuclear membrane, some of them became swollen and many contained granules and nuclear débris. The formation of cytoplasmic blebs which detached themselves as dark structureless spheres was always a prelude to the death of the cell.

In the best preparations about 80 per cent of the lymphocytes were alive and motile after incubation for 24 hr. and about 20 per cent after 48 hr. A few motile lymphocytes were still present on the 5th day but none ever survived to the 6th day. No large or medium lymphocytes were alive after 36 hr. The survival-time of the small lymphocytes showed a considerable variation from experiment to experiment and this could not be explained. The substitution of the rat's own serum for the rabbit serum in the culture medium did not affect the survival-time of the lymphocytes. No significant difference was noted between the behaviour of the lymphocytes in lymph collected from the pump outlet and in lymph collected directly from

the thoracic duct. It was inferred that the cells were not damaged by passing through the pump and that they were certainly alive when they entered the rat.

Intravenous re-infusion of lymph and killed lymphocytes

The lymphocytes were killed by circulating the lymph through a coil of polythene tubing which was either irradiated with ultra-violet light or heated in a water bath. The coil was inserted into the pumping circuit between the rat and the outlet of the pump.

The ultra-violet apparatus is illustrated in Fig. 2. A 15 cm. length of polythene tubing was coiled on the front of a cylindrical quartz-glass vessel. The back of this vessel projected into a metal box which housed a low pressure mercury vapour lamp. Cold water was circulated through the quartz vessel to cool it. The lymphocytes were heat-killed by immersing the coil of polythene in a water bath at either 56° or 45°.

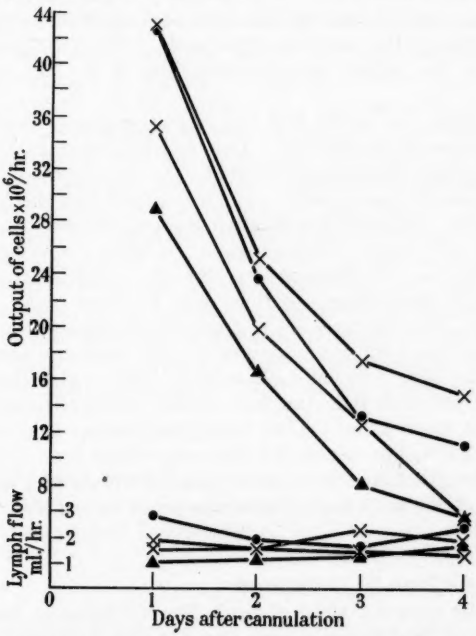

FIG. 19.—Output of lymphocytes and rate of lymph flow from the thoracic duct in 4 rats. Mean values per hr. for each successive 24 hr. after cannulation. The rats received a continuous intravenous re-infusion of lymph containing dead cells. The cells were killed by exposure for 1 min. to ultra-violet light ×———×, to heat (56°) ●———●, and to heat (45°) ▲———▲.

The lymph in the coil was exposed to ultra-violet light or to heat for 0·5 to 4 min. depending on the pumping rate ; the average exposure time during an experiment was about 1 min. An exposure for 1 min. to either 45° or to ultra-violet light did not kill all the cells immediately, a proportion of them were motile for a while when examined *in vitro*, but they were all dead after about 7 hr. incubation (Fig. 3). At this time almost all the cells in control cultures were motile (Fig. 4).

Intravenous re-infusion of cell-free lymph

The lymph was centrifuged and the cell-free supernatant was pipetted into the reservoir and returned by the pump to the rat. At night, the slowest pumping rate was employed and

the lymph was collected into a sterile flask which contained Hanks' solution and heparin. The centrifuged overnight collection (about 7 hr.) was easily returned to the rat the following morning by pumping at the fastest rate for a while. By varying the pumping rate in this way all the lymph, deprived of its cells, was re-infused into the rat.

RESULTS

The cells in rat thoracic duct lymph

In stained films about 95 per cent of the cells in rat thoracic duct lymph were typical " small " lymphocytes. The larger cells could roughly be divided into " large " and " medium " lymphocytes but the distinction was not rigid and cells of all intermediate sizes were seen. The intensity of the basophilic staining of the cytoplasm was very variable among the large and medium lymphocytes. It was most intense in some of the medium-sized cells. Mitotic figures were seen in a small proportion of the larger lymphocytes (Fig. 9–11) but never in the small lymphocytes.

In the living state, the small and large lymphocytes closely resembled each other in appearance and movement. This is illustrated in Fig. 5–8 where the shapes assumed by two large lymphocytes can be compared with those of the small lymphocytes in the same field. Both types of lymphocyte can be seen to have a dark sharply outlined appearance and a relative lack of internal cell detail. These features readily distinguished the large lymphocyte from the very rare macrophage seen in rat thoracic duct lymph. The continual drifting of the large lymphocyte in the culture medium was another point of distinction, for unlike macrophages, lymphocytes do not adhere to glass surfaces (Harris, 1953).

Many large and medium lymphocytes in the cultures were seen to divide especially during the first 6 hr. of incubation. Fig. 12–15 illustrate the division of a large lymphocyte into two daughter cells. Individual daughter cells were watched for periods up to 12 hr. They remained smaller than the parent cell and became actively motile, but a cell division was never seen in them before they died. The small lymphocytes were never seen to divide and although individual cells lived for periods up to 5 days, there was no evidence that they changed into any other cell type.

Output of lymphocytes from the thoracic duct

Table I gives the data for the 1st day's flow of thoracic duct lymph from 27 rats. The output of lymphocytes was found to be independent of the rate of lymph flow and of the body weight of the rat in the ranges given.

Assuming a blood volume of 5 ml. per 100 g. of body wt. (Griffith and Campbell, 1937) and calculating from the mean figures in Table I, the total number of lymphocytes in the blood ($68{\cdot}8 \times 10^6$) would be replaced about 11 times daily by an output from the thoracic duct of 768×10^6 cells per day.

TABLE I.—*Lymph Flow and Output of Lymphocytes from Thoracic Duct during First 24 hr. after Cannulation*

	Body wt. (g.)	Pre-operative blood lymphocytes ($\times 10^3$/cu. mm.)	Mean lymph flow (ml./hr.)	Total output of lymphocytes ($\times 10^6$)	Mean hourly output of lymphocytes ($\times 10^6$)
Mean values for 27 rats	172	8·0	1·6	768	32·0
Range	145–200	5·3–12·2	0·7–2·7	547–1100	22·8–45·8

The effect of draining lymph for 7 days on the output of lymphocytes from the thoracic duct is shown in Fig. 16. The hourly output of cells was fairly constant during the 1st day and sometimes for part of the 2nd, but a sharp fall always occurred during the 3rd and 4th days. This fall was independent of the rate of lymph flow. The output levelled off around the 5th day at 20–30 per cent of the initial value ; it remained fairly constant at 2×10^6–6×10^6 cells per hour between the 7th and 14th day after cannulation. The greater part of the fall in the output had occurred by the end of the 4th day, and where the effect of intravenous re-infusion was studied, all the experiments were terminated at this time.

The effect of continuous intravenous re-infusion of lymph and lymphocytes

Fig. 17 and Table II show that the output of lymphocytes from the thoracic duct of rats in which lymph and living lymphocytes were continuously re-infused remained almost constant for the 4 days of each experiment. The output in rats which received no re-infusion of cells or lymph is included for comparison.

The fall in output when cells and lymph are lost from the animal remains to be explained. This fall was not due to loss of lymph fluid because the continuous re-infusion of cell-free lymph did not prevent it (Fig. 18 and Table II).

It was thought that the rapid loss of cells from a thoracic duct fistula might deprive the animal of materials essential for the synthesis of new lymphocytes. If the death of lymphocytes in the animal continually supplies substances which are re-utilized in the formation of new cells, then the re-infusion of lymph and killed lymphocytes might be expected to maintain the output from the thoracic duct. However, the continuous re-infusion of lymph which contained lymphocytes killed either by ultra-violet light or by mild heat did not prevent the fall in output (Fig. 19 and Table II).

TABLE II.—*Body Weight, Blood Lymphocyte Count and Output of Lymphocytes from Thoracic Duct over 4 Days, with and without Continuous Intravenous Re-infusion of Lymph and Lymphocytes*

Intravenous re-infusion :	Rat No.	Body wt. (g.)			Blood lymphocytes ($\times 10^3$/cu. mm.)			*Mean hourly output of lymphocytes ($\times 10^6$) from thoracic duct during :		
		Pre-op	t.d.	Per cent change	Pre-op	t.d.	Per cent change	1st day	4th day	Per cent change
None	94	178	137	−23	5·3	2·5	−53	31·2	5·2	−83
	97	162	132	−19	10·2	4·2	−59	27·6	6·7	−76
	104	181	150	−17	11·4	1·9	−83	22·8	4·5	−80
Cell-free lymph	52	173	—	—	8·0	—	—	39·6	11·4	−71
	112	162	151	−7	7·1	3·5	−51	45·8	9·8	−79
	113	159	143	−10	7·0	2·0	−71	41·0	11·5	−72
Lymph and killed cells	106	161	140	−13	8·7	5·6	−36	42·5	10·9	−74
	108	158	146	−8	9·2	4·4	−52	28·8	5·6	−81
	109	150	136	−9	8·3	3·9	−53	35·2	5·6	−84
	110	182	170	−7	7·8	2·2	−72	43·0	14·9	−65
Lymph and living cells	98	133	141	+6	8·9	11·6	+30	28·8	27·3	−5
	102	200	192	−4	5·5	8·3	+51	30·6	32·1	+5
	105	158	157	−1	7·2	6·3	−13	28·2	24·0	−15

Pre-op = pre-operative value. t.d. = value at end of fourth day after cannulation.

* The consecutive daily outputs from these 13 rats are recorded in Fig. 17, 18 and 19.

Changes in body weight and in the blood

The only rats in which body weight, blood lymphocyte count and level of lymphocyte output from the thoracic duct were increased or remained almost unchanged during the four days of the experiment were those in which lymph and living lymphocytes were continuously re-infused (Table II). Rats with no re-infusion of cells or lymph became dehydrated as shown by their high haemoglobin and haematocrit levels at the end of the experiment. The other groups lost less weight and had substantially normal haemoglobin and haematocrit values. All the animals developed a polymorphonuclear leucocytosis by the end of the experiment, the counts varying from 5000 to 20,000 cells per cu. mm. This remains unexplained. The rats in all groups were lively and well when removed from the cage after four days.

DISCUSSION

Hall and Furth (1938) suggested that the large and medium lymphocytes in rabbit thoracic duct lymph divided to produce small lymphocytes although the complete sequence of change in individual cells was not observed. In the present experiments, where cultures were prepared from rat thoracic duct lymph, the products of the division of the large and medium lymphocytes remained intermediate in size between the parent cell and the small lymphocyte. Individual daughter cells were watched for periods up to 12 hours but no further division was seen in them before they died. Although the formation of small from large lymphocytes was not seen in these cultures, a comparison *in vitro* of the movements and structure of the small, medium and large cells in rat thoracic duct lymph strongly suggests that these cells belong to the same lineage. The small lymphocytes in the cultures did not divide and their morphology and type of movement remained unchanged for periods up to 5 days. Hall and Furth (1938) and Medawar (1940) also failed to observe cell transformations in small lymphocytes in cultures of thoracic duct lymph from the rabbit. In view of the claims that small lymphocytes can undergo transformations both *in vitro* (Trowell, 1955) and *in vivo* (Rebuck and Crowley, 1955), it can always be objected that the failure to observe them in the present experiments was due to the absence of the correct conditions. However, it must be emphasized that the crucial experiment of watching an individual small lymphocyte as it changes into another cell type has not been done.

The mean output of 768×10^6 lymphocytes per day from the thoracic duct of unanaesthetized rats agrees well with the figures published by Mann and Higgins (1950). It is lower than the output reported by Whaler and Widdicombe (1956) who calculated that the blood lymphocytes in young rats would be replaced 17·8 times daily by the output from the thoracic duct ; a daily replacement factor of about 11 was obtained for the rats in the present series. Thus, on the basis of the thoracic duct output in the rat, the average time spent by lymphocytes in the blood is at the most, 2 hours. This is certainly an over-estimate since the thoracic duct is not the only source of blood lymphocytes.

The present experiments confirmed the finding of Mann and Higgins (1950) that a profound fall in lymphocyte output from the thoracic duct occurs when drainage of lymph is continued for more than 2 days. It was found that this fall in output could be prevented by the continuous intravenous re-infusion of lymph and living lymphocytes : the blood lymphocyte count and the output of thoracic

duct lymphocytes were increased or remained almost unchanged during the 4 days of each experiment. From this it is inferred that the first day's output of cells, on which estimates of the rate of lymphocyte turnover in the blood have been made, probably represents the physiological output in the intact animal.

The intravenous re-infusion of cell-free lymph did not prevent the fall in lymphocyte output and it appeared that the continuous entry of lymphocytes into the blood was essential for maintaining the output of lymphocytes from the thoracic duct.

The way in which the re-infused cells exerted their effect was not clear. It was thought that the chemical components of dead lymphocytes might be continually re-utilized in the animal for the formation of new cells. The experiments in which the re-infusion of killed lymphocytes failed to maintain the thoracic duct output do not rule out this explanation, although they make it unlikely. The methods used for killing the lymphocytes were as " gentle " as could be devised but it is still possible that they may have destroyed substances in the cells which were essential for new lymphocyte production. Further, it is possible that the products of dead lymphocytes must be deposited in the correct place (*e.g.*, in the lymph nodes) for them to exert their effect. The intravenous re-infusion of dead cells might not meet this requirement.

The most attractive interpretation of these experiments is that the entrance of *living* lymphocytes into the blood is essential for maintaining the output of lymphocytes from the thoracic duct. If this is accepted, then the possibility must be considered that the re-infused lymphocytes exerted their effect by leaving the blood and returning to the lymph nodes or to the lymphatic vessels. The output from the thoracic duct might then be maintained in three ways. First, the lymphocytes might die in the lymph nodes and release in them substances necessary for new cell production. Certainly cell débris is often seen in lymph nodes. Second, large numbers of lymphocytes might leave the blood and eventually re-enter the thoracic duct *viâ* the lymph nodes or *viâ* the tissue spaces. Yoffey and Drinker (1939) have criticized the notion of a lymphocyte recirculation through the tissue spaces on the grounds that peripheral lymph (*i.e.*, lymph that has not yet passed through a lymph node) contains very few cells. However, thoracic duct lymphocytes in the rat come almost exclusively from the intestinal duct (Mann and Higgins, 1950) and the conclusions of Yoffey and Drinker were based largely on cell counts on peripheral lymph collected from the limbs. Third, the lymph nodes might become re-populated from the blood by a small proportion of lymphocytes which are capable of cell division. Rat thoracic duct lymph contains about 5 per cent of large and medium lymphocytes which can be observed *in vitro* to divide. Possibly these are the cells which Mann and Higgins (1950) suggested might be responsible for maintaining lymphocyte production. At the moment, too little is known about the life history of the lymphocyte to decide among these possibilities.

SUMMARY

The large lymphocytes in cultures of rat thoracic duct lymph were seen to divide. No cell division or cell transformation was seen in the small lymphocytes although they remained alive in the cultures for periods up to 5 days.

The daily output of lymphocytes from the thoracic duct of unanaesthetized rats was sufficient to replace all the lymphocytes in the blood about 11 times daily.

An apparatus is described for the continuous intravenous re-infusion of lymph and lymphocytes into unanaesthetized rats with thoracic duct fistulae.

It was confirmed that a profound fall in lymphocyte output from the thoracic duct occurs when drainage of lymph is continued for more than 2 days. This fall in output could be prevented by the continuous intravenous re-infusion of lymph and living lymphocytes. The continuous re-infusion of either cell-free lymph or of lymph and killed lymphocytes was ineffective.

It is suggested that the continuous entry of living lymphocytes into the blood may be essential for maintaining the output of lymphocytes from the thoracic duct.

I should like to thank Professor Sir Howard Florey, F.R.S. for his advice and encouragement. My thanks are also due to Mr. M. J. Alderman and Mr. G. J. Wren for valuable technical assistance, to Mr. H. J. Stroud and Mr. A. D. Broadway for making the apparatus, to Mr. F. Bradley for the photographs and to Miss C. Court for the drawing.

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THE VALUE AND DURATION OF DEFENCE REACTIONS OF THE SKIN TO THE PRIMARY LODGEMENT OF BACTERIA

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In all animal infections there is a stage when the infecting microbes, taking advantage of a breach in the surface defences of the host, make their primary lodgement in or on the tissues. The fate of this primary lodgement—its suppression or its persistence and growth into an established infection—is determined in part by the antimicrobial properties of the tissues immediately round it. Little, however, is known of the efficiency of these tissue responses in the early stages of infection, of the part they play in non-specific immunity, or of the physiological and pathological defects in the responses that determine the breakdown of natural resistances to the primary lodgement. Experimentally, it is obvious that suppression of this kind occurs when sub-infective doses of living pathogens are injected; and in nature they may justifiably be assumed to occur as part of the means whereby animals remain healthy in spite of the substantial microbial hazards of their environment.

We have investigated the local defence reactions in the skin of the guinea-pig, and have attempted to measure their efficacy in nine different infections. Our methods are based on the results of a study of the enhancement of infection by local adrenaline (Evans, Miles and Niven, 1948) and shock (Miles and Niven, 1950). The initiation in the guinea-pig of 2 hours' local adrenaline ischaemia or of 2–3 hours' general dehydration shock, at the time of intracutaneous injection of certain bacteria, substantially increased the maximum size attained by the local infective lesion. The increase was equivalent to multiplying the infecting dose by a factor of 10 to 10^6, according to the pathogen used. When, however, the bacterial lesions were 3–4 hours old, the induction of general shock modified the local infection either little or not at all. Such results suggest firstly that the defences inhibited by shock have a high protective value in an untreated lesion, destroying all but 10^{-1} to 10^{-6} of the infecting dose ; secondly, that they are effective mainly within the first few hours of making the primary lodgement ; and thirdly, that during this period they are decisive in determining the full extent of the local infection (Miles, 1953–54, 1956). We now present evidence, based on a study of a greater variety of bacteria and of agents which modify infection, that this early decisive period of antimicrobial reactions is common to a number of experimental infections of the skin.

MATERIALS AND METHODS

" Liquoid " (Hoffmann—La Roche) is sodium polyanethol sulphonate ; Heparin B.P. was used and assumed to contain 90 units per mg. Streptomycin was used as the sulphate, the preparation containing 780 units/mg., and the penicillin was Benzylpenicillin B.P. Shock lasting 3–4 hr. was induced by the intraperitoneal injection of 16–17 ml. 60 per cent glucose/kg. body weight (Miles and Niven, 1950).

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Bacteria.—The strains were *Streptococcus pyogenes* Group C, CN771 of the Wellcome Collection; *Staphylococcus aureus*, Au 21 from Professor Wilson Smith and CN491 of the Wellcome Collection; *Corynebacterium diphtheriae*, var. *mitis*; *Corynebacterium ovis*, Bl/50 from Professor H. R. Carne; *Listeria monocytogenes*, L71 from Professor E. G. D. Murray; *Clostridium welchii*, S.R.9; *Pseudomonas pyocyanea*, "Scudder"; *Bacterium coli*, 88; *Proteus vulgaris*, PR3 (Miles, 1951). They were preserved in the dry state by the method of Stamp (1947).

Guinea-pigs.—Albino guinea-pigs weighing 300–600 g. were used; in any one experiment, the range of weights was usually within 50 g. The skin was depilated as described by Miles and Miles (1952).

Titration of infectivity.—The bacterial growth from 18–20 hr. culture in nutrient or 5 per cent blood broth at 37° was spun down and re-suspended in a volume of liquid equal to that of the discarded supernatant medium. When required, viable counts were made by the method of Miles and Misra (1938). The bacteria in suspension usually remained fully viable in saline (0·85 per cent) for several hours, but when the bacteria died rapidly in saline, saline containing 0·1 per cent peptone or 0·02 per cent purified guinea-pig serum albumin (fraction AP3, Wilhelm, Miles and Mackay, 1955) was used. With both these stabilizers the viable count was constant for several hours in suspensions held at 4°. The albumin is preferable, because unlike peptone, it is non-toxic to the capillary endothelium of the guinea-pig.

Serial 5- or 10-fold dilutions of the suspension were injected intracutaneously in 0·1 ml. volumes; up to 24 infective lesions are readily accommodated on a 350 g. animal. The injection sites were partly randomized over the skin of the trunk, in the rectangular area posterior to the scapula and anterior to the knee joint in the sitting animal, and superior to a line 3–4 cm. on each side of the ventral mid-line. Each dose of bacteria was tested in 3–6 animals, sometimes in duplicate in each animal; the diameters recorded are the means of those from 3–12 lesions.

All nine test bacteria made lesions which reached a maximum size after 20–36 hr., except *C. ovis*, whose lesions slowly increased in size for 2 more days; and, with the exception of the two corynebacteria, none of the strains in the doses used induced a progressive generalized disease. Intracutaneous *C. ovis* and *C. diphtheriae* killed by toxaemia in 3–4 days. Animals given *C. ovis* showed no sign of intoxication after 1 day, when they were usually killed; animals given *C. diphtheriae* were protected against intoxication by 10–50 units antitoxin/kg. intraperitoneally 4–8 hr. after infection.

The diameter and thickness of the region of indurated and erythematous tissues and the diameter of any area of necrosis, were measured after 20–24 hr. Areas of erythema only, which often surround the indurated area, are usually readily distinguished, because the guinea-pig reacts to handling during measurement by a blanching of the skin, during which the peripheral erythema becomes inconspicuous. Most lesions were circular and their diameters were measured to the nearest 0·5 mm. The diameter of elliptical lesions was recorded as $\sqrt{Dd}$, where D and d are the lengths of the major and minor axes. With very skew, heavily enhanced lesions, the extent was outlined on cellophan, the area (A) measured directly on squared paper, and $\sqrt{A}$ recorded as lesion diameter. These maximum or near-maximum lesions did not change much in size between 17 and 25 hr., so that it was possible to make the final definitive measurement of lesion size at one time on the day after experiments in which lesions were initiated as much as 7 hr. apart.

Lesions in the 7–15 mm. range represent a moderate infection, usually ending in central necrosis, the diameter of the necrotic area being up to 30 per cent that of the indurated area. There was usually a feebler, though a well-defined, induration in lesions of 4–7 mm., and useful comparisons were sometimes made in this range. Lesions ranging from 15–30 mm. or more in diameter were less easy to measure precisely because they were usually severely necrotic, and sometimes grossly elongated in shape, with the long axis pointing downwards in the sitting animal, as though their eccentric spread had been determined by gravity.

RESULTS

The Dosage-response to Pathogenic Bacteria

Plotted against log dose of bacteria, neither diameter, area, nor volume of 24-hr. lesions, nor any of the valid biometrical transformations of these values, fell on a straight line with any consistency over the whole range of easily measur-

able responses, namely of lesions 5–25 mm. in diameter. With most of the test bacteria, however, lesion-diameters between 7 and 15 mm. increased in a roughly linear manner when plotted against log dose, and the response lines were roughly parallel to one another (*cf.* Miles, 1954). We therefore worked as much as possible in this range, and adopted 10 mm. as the standard response at which to compare infectivity. Thus the "skin virulence" of the test pathogens themselves is recorded in col. 1, Table I, as the ED_{10}, the effective dose producing this standard 10 mm. lesion. The comparison at the 10 mm. level of infectivity, of a given pathogen under different conditions, is exemplified in Fig. 1. The slope of the

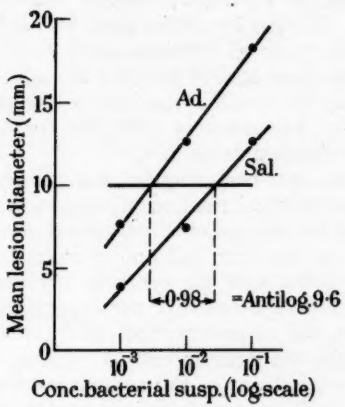

FIG. 1.—Enhancement of *C. diphtheriae* infections by 2 μg. adrenaline (Ad). Sal. = control lesions by bacteria suspended in saline.

C. diphtheriae lesions enhanced by adrenaline, as fitted by eye, is 0·98 distant, on a logarithmic scale, from that of the untreated lesions, and the enhancement is therefore equivalent to an increase in dose of antilog 0·98 = 9·6-fold, an enhancement factor (F) of about 10.

Strictly speaking, the degrees of enhancement or depression determined at the 10 mm. level are valid only for responses in that range ; the conditions under which they are valid are further restricted by the variability of slope from test to test. Nevertheless, the results of repeated comparisons at this and other levels were sufficiently consistent to justify generalizing from them, provided not too much weight is put upon the actual numerical values obtained. In any single experiment, a 5-fold difference in dose was certainly significant, and where smaller differences are cited as significant, they were established by repeated tests.

Although the values for the slope of the dosage-response in the 7–15 mm. range varied from test to test, they clustered round a characteristic figure for each bacterium. Most of the characteristic slopes were similar, lying between 2·3 and 3·0 (Table I, col. 2) ; but that for *L. monocytogenes* was shallower (1·5), and that for *Cl. welchii* steeper (3·4), than the rest. These characteristic values were used in estimating the enhancement of infectivity in experiments where only a single concentration of bacteria was used. Thus staphylococcal lesions in

shocked animals were on the average 4·1 larger than those in the control animals. The *Staph. aureus* slope being 2·3 (col. 1), the enhancement is equivalent to an increase in dose of antilog 4·1/2·3 = approx. 60-fold.

Modification of Experimental Infections

Three properties of each modifier were determined : direct action, duration of effect on the tissues, and efficacy in infected lesions of different ages.

Local modification.—For direct action, the modifier is mixed with the washed bacteria a few minutes before injection ; control injections of bacteria + saline are also made (*cf.* Fig. 1). For the two other tests we used the technique of super-injection (Miles, 1949). In tests of duration, each animal receives a number of 0·1 ml. injections of saline alone and of modifier in saline, at 0, 1, 2 . . . 6 hr. The puncture hole made by the needle is marked by a trace of India ink previously painted on the needle tip. Immediately after the last injection, when the skin bears saline- and modifier-treated areas 6, 5, 4 . . . 0 hr. old, 0·1 ml. bacterial suspension is super-injected into each original puncture hole (*cf.* Fig. 4). In tests of lesions of different ages, several marked injections of bacterial suspension are made at one time, and as the lesions reach the required age, two of them receive super-injections, one of the modifier and one of saline (*cf.* Fig. 5). In the last two tests, with lesions of varied ages, the controls, consisting of sites injected with saline, are required throughout the course of the experiment, because the mechanical disturbance made by the super-injection of 0·1 ml. of liquid may modify bacterial lesions to a slight but definite extent varying with the bacterium and the age of lesion on super-injection (*cf.* the control lesions in Fig. 5B, F).

Systemic modification.—When agents affecting the whole animal are used, their direct action, duration of effect and relative efficacy on infections of different ages are determined in a single test. Bacterial suspension is injected at intervals during a 6 or 7 hr. period into each animal of a batch. The systemic modifier is given to half the animals when the earliest lesion is about 3 hr. old, so that by the time the latest lesions are initiated, the modifier has been acting for 3–4 hr. The curves in Fig. 2 exemplify this test. The lesions initiated 1 hr. after the intraperitoneal glucose are maximally enhanced by the shock (*b*). The mean difference between control and test lesions at this point is 4·1 mm., and as noted above, the enhancement corresponds to about a 60-fold (antilog 4·1/2·3) increase in dose. The shock is effective a little over 1 hr. after the maximum (*b* to *c*), and lesions 2 hr. old when the shock was initiated (*a*) are insusceptible to its enhancing action (see also Fig. 8).

In these examples, the full range of lesion ages was tested in each animal. The procedure is justified when all the corresponding lesions in the control animals are similar in size. In some infections, however, it was evident in the control animals that the earlier lesions had induced a substantial general resistance of the skin to the same suspension injected at the 4th to the 7th hour, so that the diameters of these later lesions, when measured at 24 hr., were smaller than those of the earlier. This has occurred to a mild degree in the experiment in Fig. 2. The change can be allowed for by a direct comparison of the control and test at each point in the time-course. However, in most cases where it occurred, definitive tests were made on a number of separate batches of animals, in which each animal bore lesions differing in age by 2 hr. at the most. This method is more laborious because of the

large number of animals needed to compensate for the inter-batch variation in animal susceptibility which is thereby introduced into the error of the assay of infectivity.

Bactericidal power of the blood.—Guinea-pig blood obtained by heart puncture was defibrinated, filtered through a little gauze to remove any particles of fibrin, and immediately distributed in 0·95 ml. volumes for the test. Ten-fold dilutions of washed bacterial suspension in saline, 0·05 ml., were added to two sets of tubes. The bactericidal action of the first,

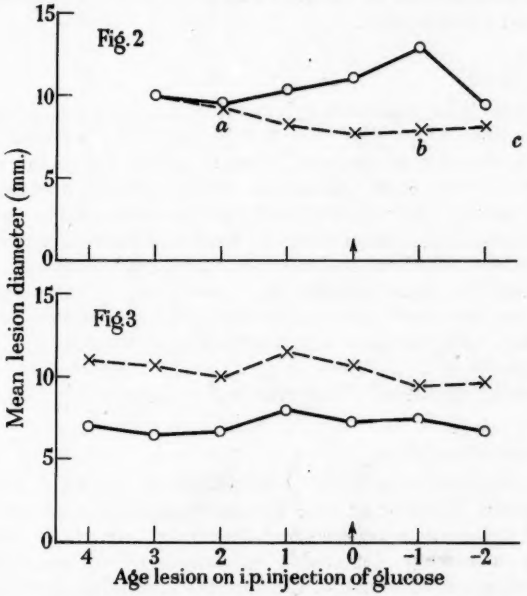

FIG. 2.—Enhancement of *Ps. pyocyanea* infections by dehydration shock, and insusceptibility to enhancement of infections initiated 2 hr. before the shock. Arrow indicates the intraperitoneal injection of glucose.
O———O Test lesions. × — — — × Control lesions.

FIG. 3.—Depression of *P. vulgaris* infections by dehydration shock. The dotted line indicates control lesion diameters. Arrow indicates the intraperitoneal injection of glucose.
O———O Test lesions. × — — — × Control lesions.

control, set was immediately destroyed, and the corpuscles laked, by adding 1 ml. 0·1 per cent Liquoid in distilled water. The second set of blood-bacterium mixtures were agitated for several hours in a water bath at 37°, in open tubes lightly plugged with cotton wool. The incubation period was determined for each bacterium, as the time at which maximum killing occurred, and proved in most cases to be 2 hr. and in others 3 or 4 hr. After incubation, the test mixtures, like the control, were treated with Liquoid solution before plating. The bactericidal power of the blood was estimated by the method of Miles and Misra (1938) from the results of 6-plaque surface plate counts made of the control and the incubated blood-bacterium mixtures; it is expressed (Table I, col. 11) as p, the proportion of the inoculum killed by the blood, total killing being unity. Each bacterium was tested on two or more occasions, and in all cases, blood and serum from the same sample were tested in parallel, to distinguish the contribution of cellular and soluble elements of the blood to its bactericidal effect on a given pathogen.

Other techniques, of experiments incidental to the main investigation, are described in the appropriate sections.

Direct Enhancement of Infection

It is impracticable to display the detailed results of the 19 definitive tests made with each of the nine pathogens. Most of the tests were repeated once, and some of them several times, each time using an average of 6 guinea-pigs. The main results are summarized in Tables I and IV ; and the details of each kind of test are illustrated by examples.

Enhancement by shock

Seven of the nine infections were enhanced by dehydration shock. The duration (D) of the enhancement (*cf.* Fig. 2) is 2–4 hr. from the injection of the glucose. After this period, the skin recovers its normal resistance to the infection (Table I, col. 3). As already reported (Miles and Niven, 1950), infections by *Cl. welchii* are strongly enhanced ; the value 10,000 for the enhancement factor F (Table I, col. 4) is very approximate, because, as in most instances of strong enhancement, the dose-response lines are skew, and the dose-ratios from enhanced and unenhanced lesions are difficult to estimate with any precision. *L. monocytogenes* and *Ps. pyocyanea* and the two cocci are moderately enhanced and *C. ovis* is enhanced only 10-fold. *Bact. coli* infections are recorded as enhanced, but in some tests of these and in all tests of *P. vulgaris* (Fig. 3) and *C. diphtheriae* infections, shock *diminished* the lesion-diameter. The reason for this effect is obscure.

Enhancement by local modifiers

Three local modifiers were used : adrenaline, 20 μg./ml.; Liquoid, 500–1000 μg./ml.; and heparin, 300–500 μg./ml. The corresponding amounts in the standard 0·1 ml. injection volumes, 2, 50–100 and 30–50 μg., are about twice the minimal doses producing a readily detectable enhancement of susceptible infections. Since in these concentrations none of the agents stimulated or inhibited the growth of the nine test pathogens in culture, it may be assumed that they enhance by direct action, not on the bacteria, but on the tissue defences. As the values of the factor (F) of enhancement show (Table I), many of the pathogens take striking advantage of this inhibition of defences.

Adrenaline.—Adrenaline is active only for a short period. The duration of its effect (D, Table I, col. 4) is 1–2·5 hr., after which the skin largely, and in most cases completely, recovers its normal resistance. For example, the streptococcus can take advantage of the change induced by 4 μg. adrenaline for 2·5 hr. after its injection ; and 0·8 μg. is effective only during the first hour (Fig. 4b, c). With *Cl. welchii* infections, D was notably short—about 40 min. (Fig. 4a), although the visible ischaemia of the skin persisted for 2 hr. after injection of the adrenaline. Infections by *Cl. welchii*, *Bact. coli* and *P. vulgaris* are strongly enhanced, and those by *C. ovis* and *L. monocytogenes* relatively feebly so ; the rest are intermediate (Table I, col. 6).

Liquoid.—The duration (D) of the Liquoid effect varied between 1 and 3 hr., except with the *Str. pyogenes*, where it was $>$ 6 hr. Although the main enhancing effect has gone after about 3 hr., a small residual increased susceptibility as a rule remains at the site for some hours after the Liquoid treatment. The highest

TABLE I.—*The Infective Dosage-response in Guinea-pig Skin to 9 Bacteria; the Factor (F) by which Infections are Enhanced by 4 Agents and the Duration of Action (D) in Hours of the Agents; and the Death Rate (p) of the 9 Pathogens in Guinea-pig Blood and Serum.*

Test pathogen	Dose response in skin: Approx. slope	Dose response in skin: ED_{10}	Enhancement by: Shock D	Shock F	Adrenaline (2 μg.) D	Adrenaline (2 μg.) F	Liquoid (50–100 μg.) D	Liquoid (50–100 μg.) F	Heparin (30–50 μg.) F	Death in: Blood p	Death in: Serum p
Str. pyogenes Group C	3·0	2×10^6	4	50	2	20	6	160	4	0·70	0·0
Staph. aureus	2·3	2×10^7	4	50	2·5	50	nt	200	1	0·83	0·0
C. diphtheriae, var. *mitis*	2·8	5×10^4	—	(–ve)	1	10	3	3–10	1	0·66	0·0
C. ovis	2·3	5×10^4	2	10	1	2	nt	10	1	0·0	0·0
L. monocytogenes	1·5	1×10^4	2	135	1	2·4	1	20	150	0·50	0·0
Cl. welchii	3·4	5×10^7	3	10^4	0·5	10^5	3	40	1	0·968	0·954
Bact. coli	2·3	1×10^9	2	10	1	10^4	nt	80	1	0·9996	0·9996
P. vulgaris	2·6	5×10^8	—	(–ve)	2	1000	2	1600	1	0·916	0·9995
Ps. pyocyanea	2·8	5×10^6	2	60	2	100	3	10^4	75	0·982	0·846
Column	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)

nt = not tested.

value of F is recorded for *Ps. pyocyanea*. The rest of the infections are moderately susceptible (Table I, col. 7 and 8).

Heparin.—Heparin was tested less as an effective modifier than as an anticoagulant for comparison with Liquoid. In doses of 50 μg. it slightly enhances *Str. pyogenes*, and strongly enhances *L. monocytogenes* and *Ps. pyocyanea*. The rest were unaffected by doses up to 100 μg. (Table I, col. 9).

That the heparin effect is complex is evident from Table II, which records the effect of graded concentrations of heparin on the two pathogens which it enhances,

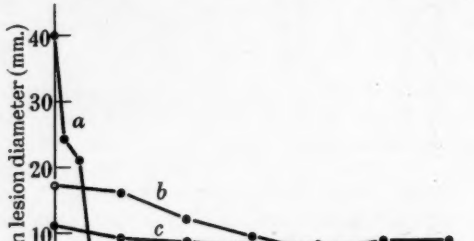

FIG. 4.—Duration of enhancing effect in skin treated with adrenaline at 0 hr.
(*a*) *Cl. welchii* with 2 μg. adrenaline;
(*b*) and (*c*) *Str. pyogenes* with 4 and 0·8 μg. adrenaline respectively.
The dotted line indicates the mean diameter of control lesions.

Str. pyogenes and *L. monocytogenes*. In both there was a striking *depression* of infection by a concentration of 6 μg./ml. Its significance is obscure, but its occurrence is a warning against too ready deductions from the effect of large doses of a modifier.

TABLE II.—*The Biphasic Action of Simultaneously Injected Heparin on* Str. pyogenes *and* L. monocytogenes *Infections of the Skin.*

Heparin (μg./ml.)	Mean lesion diameter (mm.)	
	Str. pyogenes	*L. monocytogenes*
500	**8·2**	**26·0**
167	6·1	16·0
55	6·5	14·0
18	6·8	13·7
6	*3·5*	*7·5*
2	5·5	10·3
0	5·6	11·3

Diameters of strongly enhanced infective lesions in bold type, and of significantly depressed lesions in italics.

Mode of Action of the Modifiers

The four enhancing agents are not consistently effective on all nine test pathogens. *Cl. welchii* infections, for example, are highly susceptible to shock

and adrenaline, but only moderately so to Liquoid; and *L. monocytogenes*, though well enhanced by shock and heparin, is feebly affected by adrenaline. The two pathogenic cocci are moderately enhanced by all agents, and the two corynebacteria rather feebly so. Like those of *Cl. welchii*, infections by the three Gram-negative bacilli are capable of great enhancement, and vary widely in susceptibility to the various modifiers.

The absence of any strict parallelism between the results with each infection is to be expected, since the various defences inhibited by these agents are unlikely to have the same relative importance for each pathogen. It is nevertheless suggestive that the readiness with which these pathogens are enhanced is broadly paralleled by their susceptibility to the bactericidal action of blood and serum (Table I, col. 10 and 11). *Cl. welchii* and the three Gram-negative bacilli are highly susceptible to both blood and serum, and all are capable of enhancement to 1000-fold or more. The moderately enhanced cocci and *C. diphtheriae* are next most susceptible, but are killed only by whole blood, not by serum. The feebly enhanced *C. ovis* is insusceptible to blood under the conditions of the test. If the modifiers are not inhibiting reactions directly dependent on elements of the blood, they are inhibiting bactericidal actions at least of the same kind as those of blood.

Shock appears to enhance infections because there is in the skin a temporary diminution of blood supply, and of the intravascular pressure necessary for the exudation of plasma bactericidins and of leucocytes (Miles and Niven, 1950). In these respects, of the local modifiers adrenaline most nearly reproduces the ischaemic effect of shock. It presumably shuts off the supply of blood-borne antibacterial substances, both cellular and humoral (Evans *et al.*, 1948). With its transient yet powerful effect in low doses, it is an admirable tool for exploring tissue defences.

Liquoid is a more brutal modifying agent, because even in minimally effective doses it leaves a residual diminution of local defences after the main effect has passed off. It is anticoagulant and anti-complementary, and, by virtue of its anti-complementary powers, readily inhibits the bactericidal action of both plasma and whole blood (von Haebler and Miles, 1938). Unlike shock and adrenaline, it does not inhibit the rapid exudation induced by histamine or leukotaxine, tested locally in the skin of guinea-pigs with pontamine sky blue in their circulation (see Miles and Miles, 1952); moreover, as judged in stained sections, it interfered only mildly with the leucocyte response of the tissues to the infecting bacterium. Its anticoagulant powers must be considered in relation to Menkin's (1940) view that the formation of intravascular thrombi and of an intercellular fibrin mesh-work materially contributes to the localization of infection by inflamed tissues. It is unlikely that the great increase in area of some infective lesions under the influence of Liquoid is due to the inhibition of any defensive coagulation, because heparin, in more effectively anticoagulant doses, does not enhance and has none of the high potency of Liquoid as an inhibitor of the bactericidal effects mediated by complement.

Table III records the potency ratio of Liquoid and Heparin B.P. in four *in vitro* tests made at 37°. The anticoagulant potency was measured in freshly-drawn heart blood of the guinea-pig, and the anti-complementary potency in a system containing 3 M.H.D. of guinea-pig complement and sheep R.B.C. sensitized with 2 M.H.D. of antibody. The antibactericidal action on defibrinated guinea-pig blood was compared using as test bacteria the two cocci and *Bact. coli*

TABLE III.—*The Relative Inhibiting Potencies of Liquoid and Heparin.*

Inhibition of	Ratio of minimal effective concentrations: Liquoid	Heparin
Complement	1	15
Chemotaxis	1	>30
Bactericidal power of blood (4 bacteria tested)	1	>10 to >27
Coagulation of blood	1	0·2

and *Ps. pyocyanea*. Chemotaxis of guinea-pig leucocytes, from 3-hr.-old peritoneal exudates, was observed in sealed slide-cells containing starch granules (Comandon, 1920); 330 μg. but not 110 μg./ml. of Liquoid totally inhibited the chemotaxis, which was unaffected by 1 per cent heparin. Weight for weight, heparin was about 5 times as potent as anticoagulant, and had from 1/10th to < 1/30th the potency of Liquoid in the three other respects. That is, in equal anticoagulant doses, Liquoid has more than 25 to 150 times the inhibiting action of heparin on complement, chemotaxis and the bactericidal power of blood.

Susceptibility to Enhancement and Age of Infection

In nearly all the infections, shock and the three local modifiers enhanced most actively during the first 1½ hr. after the primary lodgement. After this, the susceptibility of the lesions declined, and none of the enhancing agents had much effect after 5 hr. Fig. 5A is characteristic of the insusceptibility of streptococcal infections more than 2 hr. old to super-injected adrenaline. The other 8 infections behaved similarly, except those with *P. vulgaris* (Fig. 5B). Here the initial susceptibility to enhancement, as with the others, declined rapidly within the hour, but older lesions were strikingly susceptible to enhancement. A similar, though slight, secondary rise occurred in one of three tests with *Bact. coli*.

As with adrenaline, initial susceptibility to Liquoid enhancement as a rule declined within the first few hours of infection, but a residual susceptibility to Liquoid was maintained (Fig. 5C, D), or returned (Fig. 5E) after a short period of insusceptibility. In none of these cases, however, was the residual enhancement more than a fraction of the direct enhancement produced by simultaneous injection of bacteria and modifier. *C. diphtheriae* lesions (Fig. 5F) were most susceptible when 2–3 hr. old, but like the rest were insusceptible at the 5th hour.

The picture of enhancement by shock was clear cut; lesions initiated 2–5 hr. before the shock were unaffected by it. The example in Fig. 2 is characteristic of the seven infections susceptible to enhancement.

The Concept of the Decisive Period

The 24-hr. lesions, in terms of whose diameters the infectivity of a bacterium is measured, represent the maximum development of local infection, the extent of which is decided by defences active at the site of the primary lodgement. The increasing insusceptibility of the infection to enhancement may be interpreted in one of two ways. Either the agent is active in older lesions, but the defences it inhibits are no longer operative or, if operative, no longer decisive; or the defences in question are still decisive in older lesions, but have become topographically or pharmacologically inaccessible to the agent. If the first explanation is correct,

the period of useful activity of a given set of defences, defined as those inhibited by the particular modifier, may be determined from results like those set out in Table IV. It is almost certainly correct in shock, which deprives the primary lodgement of its supply of bactericidal elements of the blood. Other consequences of shock are theoretically possible. Thus, a systemic tissue poison might be generated, whose movement into the older lesion might be prevented by, *e.g.*, thrombosis of blood vessels, changes in permeability of the vessel walls, or extravascular events like coagulation in the infected tissues. There is, however, no evidence of such an enhancing toxin in shock. Moreover, if there were, there is

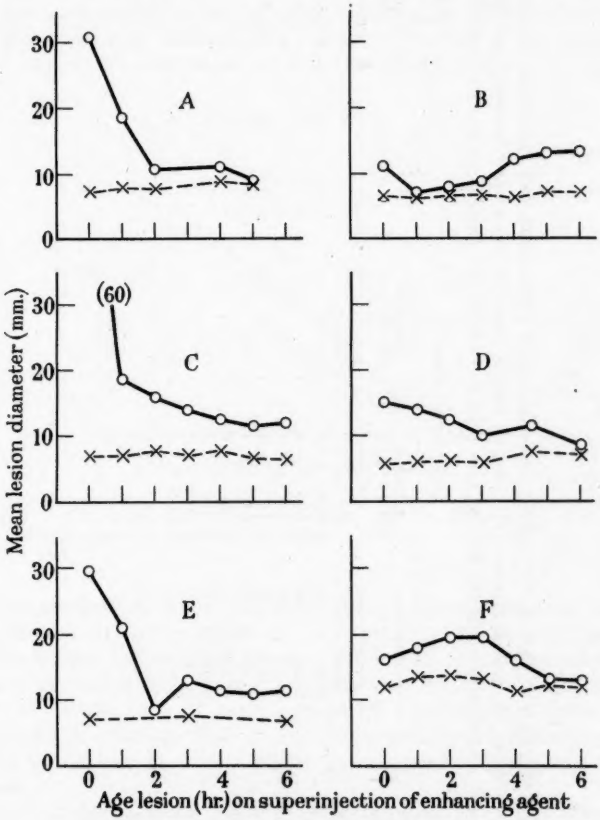

FIG. 5.—The change in susceptibility of various infections to local enhancement with increasing age of lesion.

A.—*Str. pyogenes* . Adrenaline, 2 μg.
B.—*P. vulgaris* . ,, ,,
C.—*Str. pyogenes* . Liquoid, 100 μg.
D.—*P. vulgaris* . ,, ,,
E.—*Cl. welchii* . ,, ,,
F.—*C. diphtheriae* . ,, ,,

O———O Test lesions. × – – – × Control lesions.

TABLE IV.—*The Effective Proportion (P_e) of the Inoculum in 9 Intracutaneous Infections Surviving Defences Inhibited by 4 Agents, with the Period in Hours (DP) during which these Defences are Decisive, and the Proportion Surviving Treatment with Antibiotics, with the Period (Hr.) during which the Infections are Susceptible to the Antibiotics.*

	Defences inhibited by								Bacteria inhibited by			
	Adrenaline (2 μg.)		Liquoid (50–100 μg.)		Heparin (30–50 μg.)		Shock		Penicillin 10^4 u.		Streptomycin 12×10^3 u.	
Test pathogen	P_e	DP	P_e	DP	P_e	DP	P_e	DP	P_e	DP	P_e	DP
Str. pyogenes Group C	0·05	4	0·006	2	0·25	4	0·02	2	0·1	3	0·02	>8
Staph. aureus	0·02	2	0·005	4	1·0	—	0·02	4	0·07	3	0·004	5
C. diphtheriae, var. *mitis*	0·1	4	0·1	4	1·0	—	—	—	0·05	3	0·01	4
C. ovis	0·5	1	0·1	3	1·0	—	0·1	3	0·007	3	0·0005	5
L. monocytogenes	0·42	2	0·05	1	0·006	nt	0·007	5	1·0	—	1·0	—
Cl. welchii	0·00001	3	0·025	3	1·0	—	0·00008	3	0·1	3	1·0	—
Bact. coli	0·0001	1	0·013	1	1·0	—	0·1	4	1·0	—	0·1	3
P. vulgaris	0·001	1	0·0006	5	1·0	—	—	—	1·0	—	0·01	3
Ps. pyocyanea	0·01	2	0·0001	3	0·013	2	0·017	3	1·0	—	0·02	3
Column	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)

nt = not tested.

no major hindrance to its entry into the lesion ; because no thrombosis, either of blood or lymphatic vessels, occurred in any of the nine local infections during the first 4–5 hr. and except in the centre of those lesions destined to become necrotic, there was no histological evidence of intercellular formation of fibrin (Burke and Miles, to be published).

The local modifiers, being super-injected, reach all parts of the lesion. Four-hour-old lesions appear to be as susceptible as the 0-hour-old lesions to adrenaline vaso-constriction, as judged by the occurrence and duration of superficial blanching of the skin. Moreover, continuing vaso-constriction for 1–2 hr. of the arterioles underlying 4-hr. streptococcal and staphylococcal lesions is directly observable under low power microscopy in the ear of the guinea-pig. If the first explanation of insusceptibility is to be accepted, it remains to show that the constriction impedes blood supply to older lesions. For this purpose we used a modification

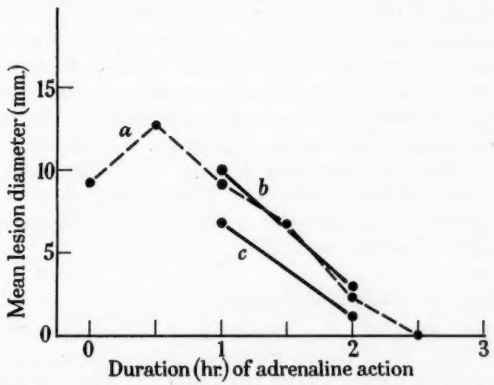

FIG. 6.—Duration of ischaemia induced by 0·1 ml. of 20 μg./ml. adrenaline, in (*a*) normal skin, (*b*) 0–hour, and (*c*) 4–hour streptococcal lesions. The diameters are of areas not filled by intra-arterial injection of a suspension of carbon.

of the India ink method of Miles and Miles (1952). Intracutaneous injections of 0·1 ml. adrenaline solution are made at intervals of 15 or 30 min. into the area of skin supplied by the left axillary artery. When the skin bears lesions 0–3½ hr. old, the artery is cannulated under anaesthesia, the returning vein opened, and a non-irritant suspension of carbon particles injected at a pressure (80–100 mg. Hg) of the same order as that in the main arteries of the animal. A dark area of skin is produced, in which white patches indicate regions supplied by occluded vessels.

The extent and duration of the occlusion induced by 2 μg. adrenaline was measured in uninfected skin and in skin bearing 0- and 4-hr. streptococcal lesions. The occluded region shrinks (Fig. 6), and in untreated skin the vessels are fully patent after 2–2½ hr. The vessels of infected skin also recover within 2 hr., when the drug and the streptococci are injected together ; and in 1½ hr., when the drug is super-injected into 4-hr. lesions. The circumstances in which the drug lasts on the average for 2 hr. are of course those of direct enhancement. In the 4-hr. lesion the ischaemia passes off a little more rapidly. However, the ischaemia

in 4-hr. lesions may be made to last for 2 hr. by using concentrations of 40–60 μg./ml. and this higher dose of adrenaline enhances such lesions either little or not at all. That is, the failure of the 20 μg./ml. to enhance 4-hr. lesions cannot in any substantial part be attributed to the relative insusceptibility of the inflamed region. Inflamed vessels are reported (Meltzer and Meltzer, 1903) as being insusceptible to adrenaline. We have no evidence on this point about individual vessels; the inflamed region as a whole certainly becomes ischaemic when the drug is injected. Some of the ischaemia is due to constriction of the larger blood vessels on and in the panniculus carnosus, which at 4–5 hr. is not conspicuously inflamed; but we cannot say whether the drug's action is confined to such vessels.

We conclude that in both shock and adrenaline enhancement, infections become progressively insusceptible because the defence reactions inhibited by these two agents become less and less decisive as the lesion ages, and are largely immaterial to the outcome of local infection after 4–5 hr.

The choice between the two explanations is more difficult to make for Liquoid enhancement. The inhibitor reaches the inflamed tissues, and there is no reason to suppose that the complement or leucocytes of the inflammatory exudate will be insusceptible to its action. But it is possible that the complement and leucocytes of a 4-hr. exudate are in excess of the amounts inhibited by the standard enhancing dose, 100 μg. of Liquoid; because on injection, 0·1 ml. of a liquid fills about 0·25 ml. volume of skin, so that the average concentration of super-injected Liquoid is of the order of 0·04 per cent; this is only about twice the minimal concentration required to destroy the relatively limited bactericidal power of defibrinated blood (von Haebler and Miles, 1938).

The decisive period of the inhibitable defences

The decisive periods (DP) for shock- and adrenaline-inhibitable defences are listed in Table IV. Each figure represents the longest period found in 2, 3 or 4 determinations. The values range from 1–5 hr. The Liquoid results are also listed under the heading DP; and although the evidence is equivocal that the period of susceptibility to Liquoid enhancement is necessarily decisive with respect to Liquoid-inhibitable defences, it is striking that these DP values, and indeed those for heparin enhancement, fall within the 5-hr. range. Of the 27 estimates of the DP, 25 are 4 hr. or less; and the mean value is 2·8 hr.

The significance of the decisive defences in the various infections depends of course on their magnitude. For example, the proportional importance of adrenaline-inhibitable defences in the course of the *Str. pyogenes* infection is much greater than in the *C. ovis* infection; the enhancement factor of the first is 50, and of the second, 2. The adrenaline treatment gave 49/50ths of the injected streptococci an opportunity to infect which was denied to them in the control lesion; that is, in untreated skin, all but 0·02 of the inoculum was killed, removed or otherwise rendered non-pathogenic by the adrenaline-inhibitable defences. The corresponding figure for *C. ovis* is 0·5.

These last values, the reciprocals of the enhancement factor F (Table I), are entered in Table IV as P_e, the effective proportion of the inoculum surviving these early defences. They clearly show that the defences are substantially effective in the nine infections. They are most effective against the Gram-negative bacilli and *Cl. welchii*, least against *L. monocytogenes* and the two corynebacteria, and intermediately so against the two cocci.

The decisive period with antibiotics

No transient stimulants—as opposed to inhibitors—of the defences were available to test the concept of the decisive period with respect to suppression rather than the enhancement of infection. But suppression of infection by antibiotics provides a means of testing whether the early period of infection is in any way as decisive for the bacteria as it is for the defences. The intravenous injection of a single dose of 10^4 units of benzylpenicillin or of 12×10^3 units of streptomycin sulphate, made 0–1 hr. before the intracutaneous injection of the bacteria, suppressed the test infections to the degree indicated by the P_e values in col. 9 and 11 of Table IV. (Since the lesions were measured at 24 hr., the late-developing toxicity of penicillin for the guinea-pig could be ignored.) Here P_e, the effective proportion of the inoculum surviving the antibiotic, is the reciprocal of the factor by which infectivity is *depressed* (*cf.* Fig. 7). Several of the infections were insusceptible to the antibiotics in the doses used and P_e therefore is unity.

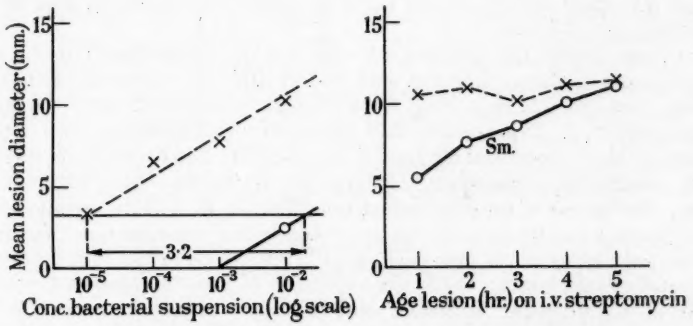

FIG. 7.—Depression of *C. ovis* infections by 12×10^3 units streptomycin, given intravenously at the time of the intracutaneous injection of the bacilli.

FIG. 8.—Decreasing susceptibility of *C. ovis* infections to 12×10^3 units streptomycin with increasing age of the bacterial lesions at the time of the intravenous injection of streptomycin.

O———O Test lesions. ×– – –× Control lesions.

The method of determining the decisive period was similar to that for shock. All but one of the infections which were susceptible when initiated at the time of the single intravenous dose of antibiotic were, as Fig. 8 exemplifies, completely insusceptible when the lesions were 3–5 hr. old. *Str. pyogenes* infection is the exception; here, lesions up to 8-hr. old continued to be susceptible to streptomycin. The decreasing efficacy of antibiotics with increasing age of infection is a commonplace of chemotherapy, but it is striking to find that the decisive period of their efficacy in a wide variety of infections coincides with that of the defences. The coincidence is consistent with what we know of the state of the lesions at the end of the decisive period. Leucocytes are plentiful, and phagocytosis in most cases is pronounced. Bacteria destined to survive the decisive period may well be intraleucocytic at this time, and to that extent protected from antibiotics arriving from the blood stream.

DISCUSSION

The nine experimental infections of the skin were surveyed as the first step of a larger investigation of non-specific resistance to microbial infection. Defence against an external microbial hazard entails either the prevention of any lodgement of the pathogen whatsoever, or its destruction in the primary lodgement before it can produce a manifest infection. The intracutaneous injection of the pathogen is equivalent to a successful primary lodgement, so that our analysis can reveal nothing about defences against the *act* of lodgement; but it reveals rapid, and in some cases highly effective, defences against bacteria newly introduced into the tissues. The exact numerical values upon which we base this conclusion are clearly to be taken as indicating only the magnitude of an effect; they are not precise measurements of it. None the less, they are reliable enough for measurement of efficacy of the body defences in orthodox terms of the dose-response to infection. On this basis it appears that, according to the pathogen used, from 50 to 99·999 per cent of the inoculum is rendered ineffective. The defences responsible for this effect come into play mainly within the first 2 hours of infection, and appear to be complete within 5 hours.

A few of our results are inconsistent with this generalization, but the striking similarity of most of them, obtained with widely differing infections and modifiers of infection, justifies our regarding it as broadly established for intracutaneous infections of this kind. It is possible that the generalization also applies to primary lodgements in other superficial regions of the body like the respiratory and alimentary tracts; and perhaps to secondary lodgements in various organs, like those occurring during the course of an established infection, as metastatic events in as yet uninfected tissue: but we have as yet no evidence on these aspects of the problem.

The test pathogens are at first sight a little remote from the microbial hazards of the natural environment. With the exception of the *Str. pyogenes*, which was isolated from a guinea-pig infection, and perhaps the *Staph. aureus*, a species sometimes infecting the skin and eyes of the laboratory guinea-pig, our test bacteria are not natural pathogens of the animal. None of them was virulent enough to infect in doses of a few cells, and only the *Str. pyogenes* and the *C. ovis* could establish a severe general infection. We contend nevertheless that they are sufficiently representative of environmental pathogens, because, except in fulminating epidemics, such pathogens are likely to be in reasonable equilibrium with a stable population of a host species—infecting a small proportion and killing a smaller one—and therefore neither highly infective nor highly virulent.

We have already noted the broad correlation between susceptibility of infection to enhancement and the susceptibility of the infecting bacterium to the bactericidal power of the blood, which suggests that the bactericidal actions inhibited by the enhancing agents are those of blood, or one of the same kind as those of leucocytes and plasma. There is also a suggestion of a broad correlation between this susceptibility and the virulence of the bacteria, as indicated by the dose producing a 10 mm. lesion in the skin (ED_{10}, Table I). It follows that the higher the ED_{10}, the greater the "kill" in the primary lodgement. Thus with *Bact. coli* the ED_{10} is 10^9 and the P_e for adrenaline is 10^{-4}, so that the estimated dose of bacilli surviving the decisive period is 10^5. *Str. pyogenes* has a lower ED_{10} of 2×10^6, and a proportionately lower P_e of 0·02, and the dose surviving the decisive period is 10^4. Most of the ED_{10}'s appear to be diminished to effective doses of the order of 10^3–10^5.

One more numerical association may be noted. Maximum killing of the bacteria in defibrinated blood occurred without exception in 2–4 hours, after which the surviving bacteria began to multiply. The decisive period of 3–4 hours, for the action *in vivo* of defences which, according to our analysis, are mediated by bactericidal elements of the blood, is about the same. The association may be coincidental, but it is also consistent with the view that the effective blood elements mobilized *in vivo* after a short period undergo no substantial renewal from the circulation and elsewhere, and in this respect resemble the sample of blood in a bactericidal test. That is, at 4 hours, the defensive responses characteristic of the first hours of the infection are no longer made.

We do not imply that the short decisive period is applicable to all local defences. It was in each case determined with a dose of modifier equal to that used to establish the enhancement factor, and is therefore valid only for defences of the degree and kind affected by the various modifiers. The modifiers were selected for their transient action and used in a few multiples of minimally effective doses. An analysis of the defences with stronger and with other modifiers might well reveal different decisive periods, but our conclusions would not be invalidated because we define the defences to which the short decisive period applies in terms of the modifiers. We have not tested other modifiers systematically, but the few so tested do not indicate another decisive period. Nevertheless, other important defence systems with different time courses are by no means ruled out. Lesions older than 4 hours are indeed modifiable. Thus adrenaline enhances 5-hour *Ps. pyocyanea* lesions (Fig. 5, B); and in the two infections *depressed* by shock (Fig. 3), lesions 6 hours old or more are as strongly affected as those initiated during shock.

The rapid initial death of a large proportion of the survivors of the inoculum and the defences already mobilized together appear to determine the final size of the local lesion. But the important events of the primary lodgement are by no means over. In the first place, though the participants in the reaction are by now decisively gathered together, the reaction is not over. A moderate oedema and erythema at 4 hours in most cases becomes a heavy induration with necrosis. In the second place, the relation of the various stages of the primary lodgement and its dissemination to other parts of the body, and the part played by systemic reactions in containing the local infection as the lesion matures, is almost entirely unknown. We have evidence that thrombosis at the periphery of the lesion plays no part in localizing it.

Lastly, we would emphasize the simplicity of intracutaneous lesions for studying infection and resistance. The information from a single depilated guinea-pig, on which 3–4 doses each of several infecting bacteria can be titrated, far exceeds that from an animal systemically infected with one kind of bacterium, from which only two simple measures of infection can be obtained—one a continuous variate, time to death, the other a quantal response, death itself. The guinea-pig yields at any one reading as many estimates of infectivity as there are lesions; and there may be 24 or more, all in a piece of tissue substantially uniform in susceptibility to the infecting agents. By contrast, titrations of equal statistical weight, using, for example, a quantal response in systemically infected mice, could be obtained only from several multiples of 24 mice. When means of the skin responses of 4–5 guinea-pigs are used, the precision of the results may be equivalent to that from several hundred mice. The intradermal method,

as will be evident from our few variations in technique, is moreover capable of great and cheaply achieved experimental flexibility.

SUMMARY

The fate of the primary lodgement of infecting microbes was studied in experimental infection of the skin of the guinea-pig by nine different bacteria.

With all nine pathogens, the mean diameters of mature lesions were roughly linear with respect to log dose, within a restricted range of dose-responses; for a given pathogen, the slopes of the dosage-response lines were roughly parallel, thus facilitating numerical comparisons of infectivity under the influence of modifying agents.

Local adrenaline, Liquoid, and general dehydration shock, enhanced nearly all the infections to a degree varying with the pathogen and the enhancing agent. In general, *Bact. coli*, *Ps. pyocyanea*, *P. vulgaris* and *Cl. welchii* were most strongly enhanced, *Str. pyogenes* and *Staph. aureus* less strongly enhanced, and *C. diphtheriae*, *C. ovis* and *L. monocytogenes* moderately enhanced.

The enhancing agents inhibit either the access to the lesion, or action in the lesion, of bactericidal elements of the blood. The degree of enhancement is in general correlated with susceptibility of the pathogen to the bactericidal action of blood and serum *in vitro*.

Enhancement ranged from 2- to 100,000-fold. The body defences inhibited by the enhancing agents are accordingly responsible for the "killing" from 50 to 99·999 per cent of the bacteria originally constituting the primary lodgement.

Infections several hours old are insusceptible to enhancement. It appears that at this stage the defences inhibitable by the enhancing agents no longer have any decisive effect on the subsequent development of the lesion. The period during which these defences are decisive varied between the first 2 and the first 5 hours of infection.

The short decisive period applies also to the bacteria in the lesions. Infections 3–5 hours old were insusceptible to intravenous antibiotics in doses that were effective when given earlier in the infection. At this stage there was no obstruction to the blood supply of the lesions.

This work was begun, and E. M. Miles' part in it completed, at the National Institute for Medical Research, Mill Hill, N.W.7. We are indebted to the Nuffield Trust for financial help in its prosecution.

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EFFECT OF TRIAC ON THE REABSORPTION OF CHOLESTEROL FROM ATHEROMATOUS LESIONS IN THE AORTA OF THE RABBIT

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TRIAC (triiodothyroacetic acid) was synthesized by Pitt-Rivers (1953) and its activity in the goitre-prevention test in rats was shown to be about one-tenth that of triiodothyronine. It was administered to two patients with myxoedema by Lerman and Pitt-Rivers (1955). Both improved clinically and the blood cholesterol was greatly reduced, but the basal metabolic rate did not increase. These observations suggested that triac might have a greater effect on the blood cholesterol level than on other aspects of metabolism, and might therefore be of use in the prevention and treatment of atheroma. Further observations by Trotter (1955, 1956) have however shown that triac can increase the basal metabolic rate in patients with myxoedema, and that, while substantial reductions in blood cholesterol can often be achieved even in euthyroid subjects, triac is not necessarily more effective in this respect than triiodothyronine or thyroxine.

Bainborough and McMillan (1952) showed that thyroxine did not hasten the rate of disappearance of atheromatous lesions induced in rabbits by previous cholesterol feeding. If triac were more effective than thyroxine in eliminating cholesterol it might be expected to give a positive result in an experiment of this type. The main difference between our observations and those of Bainborough and McMillan is that we have used the cholesterol content of the aorta as the criterion by which the effect of the drug was assessed, rather than the morphological appearances of the aorta.

METHOD

Thirty "sandy-lop" rabbits were fed on diet 18 (Bruce and Parkes, 1946) and given in addition 1 g. of cholesterol daily for 12 weeks. Serum cholesterol was estimated just before the end of the period of cholesterol feeding. One week after the cholesterol had been stopped the rabbits were divided into pairs having approximately equal serum cholesterol levels. One member of each pair was allotted to the experimental group, the other to the control. Both groups then remained on the same diet (without added cholesterol), but the rabbits in the experimental group each received in addition 200 μg. of triac daily, given orally in a 10 per cent solution of sucrose. The dose was then raised, at fortnightly intervals, until the maximum tolerated dose was found. This proved to be 300 μg. per rabbit per day, and this dose was maintained until the termination of the experiment after 26 weeks of triac administration. Two controls and one experimental animal had to be killed because of coccidiosis or middle ear infection. The remaining 27 animals were then killed by exsanguination under pentobarbital anaesthesia. The aortas were dissected out, inspected, weighed and extracted with alcohol-ether in a Soxhlet apparatus. The serum and aorta cholesterol values for three normal rabbits were also determined. Cholesterol was estimated by the method of Sackett (1925).

RESULTS

In the Figure the cholesterol content of the aorta has been plotted against serum cholesterol (estimated just before triac was started). It was found that the relationship between these two functions was most nearly linear when a logarithmic scale was used for aortic cholesterol. The regression of log. aortic cholesterol on serum cholesterol was calculated separately for the treated and control groups. Neither the slope of the regression line nor its height above the

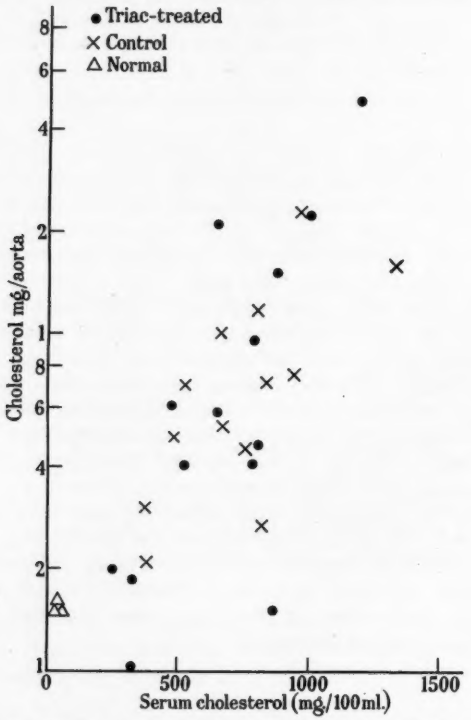

FIGURE.—Relationship between serum cholesterol (mg./100 ml.) at the start of triac feeding and aortic cholesterol (mg. per aorta) at the end of the experiment.

base line was significantly different in the two groups. It can therefore be concluded that triac had no appreciable effect on aortic cholesterol. The figures for the treated and control groups were then combined; the correlation coefficient for the combined series was +0·67 and highly significant. The demonstration of a significant correlation between serum cholesterol, before triac was started, and the log. of the aortic cholesterol at the end of the experiment, justifies the division of the rabbits into two comparable groups on the basis of serum cholesterol levels.

Inspection of the aortas confirmed that the extent of the atheromatous lesions was similar in the treated and control groups.

DISCUSSION

When given in doses which did not affect the rabbits' health or body weight, triac failed to mobilize cholesterol from atheromatous lesions. It is possible that higher doses might have been more successful.

In testing the effect of drugs in experimental atheroma the usual practice has been to administer the substance under test during the period of cholesterol feeding. The type of experiment we have used is however somewhat nearer to the state of affairs in human pathology, in that the drug under test does not have to contend with an enormous and unnatural intake of cholesterol. The success of our type of experiment depends on assessing the degree of atheroma in individual rabbits before the start of drug treatment, so that paired controls can be employed. Our observations suggest that the degree of atheroma can be assessed fairly adequately by the serum cholesterol level immediately before the end of the period of cholesterol feeding.

SUMMARY

Thirty rabbits were given cholesterol in their diet for 12 weeks. They were then divided into experimental and control groups on the basis of the serum cholesterol levels. Cholesterol feeding was stopped, and the animals in the experimental group were given 300 μg. of triac daily for 26 weeks. The cholesterol content of the aortas was not materially different in the treated and control groups. There was a significant correlation between aortic cholesterol content and serum cholesterol, estimated at the end of the period of cholesterol feeding.

We wish to thank Mr. N. W. Please for statistical help and Mrs. S. Brownstone for the cholesterol analyses.

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EFFECT OF ADRENAL HORMONES AND ADRENALECTOMY ON MITOTIC ACTIVITY*

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ALTHOUGH considerable work has been done on the effect of exogenous chemical substances which inhibit or stimulate mitotic activity, comparatively little work has been done on the effect of endogenous substances such as hormones. The effects of testosterone and oestrogens have been studied and these have been shown to stimulate mitotic activity (Bullough and van Oordt, 1950; Bullough, 1946, 1950), but the effect of the adrenal hormones had not been investigated when this work was started.

That such a study might prove worth while became apparent on reviewing the effects of adrenocorticotrophic hormone of the anterior pituitary (ACTH) and cortisone on tissue growth observed by other workers. Thus it has been shown that ACTH, which induces glucocorticoid hormone secretion, suppresses the growth of epidermis, sebaceous glands and hair (Baker, Ingle, Li and Evans, 1948) and that cortisone itself reduces the size of sebaceous glands (Castor and Baker, 1950). Further, it has been shown that ACTH causes involution of the lymph nodes (Baker, Ingle and Li, 1951; Feldman, 1951), while cortisone retards the growth rate of young rats (Wells and Kendall, 1940; Baker, 1950). Several recent studies have also indicated that the glucocorticoid hormones inhibit wound healing (Baker and Whitaker, 1950; Bangham, 1951; Billingham, Krohn and Medawar, 1951). The daily administration of cortisone delays the vascularization of a wound, weakens primary healing, depresses cellular proliferation and retards tissue reorganization. Green (1950) suggested that one possible way in which the adrenal hormones could produce such effects would be by depressing mitotic activity in the tissues.

Meanwhile a different approach to the problem began to suggest that the adrenal hormones might have an inhibiting effect on mitosis. Green and Bullough (1950) showed that following hind-limb ischaemia or the injection of adenosine triphosphate (ATP) there was an early and strong reduction, or complete suppression, of mitotic activity in the skin of the mouse. Further, it was demonstrated (Stoner and Green, 1950) that these stress-producing stimuli lead to a stimulation of the pituitary-cortical mechanism and the liberation of cortical hormones. It seemed likely therefore that the mitotic inhibition seen under these circumstances was produced indirectly as a result of the adrenal hormones, and it was decided to test this hypothesis by studying directly the effect of these hormones on mitotic activity in the mouse epidermis.

Bullough (1948*a*) devised a technique for studying mitotic activity in the ear epidermis of the mouse. He showed that there exists a clearly defined double diurnal cycle of mitotic activity in the epidermis of adult (3–4 months old) male

* This paper is a summary of a part of a thesis submitted by one of us (F. N. G.) to London University for the degree of Ph.D.

mice, when these animals are kept at a temperature of 20° and fed every day between 9 and 10 a.m. Under these conditions periods of maximum mitotic activity were seen at 6 a.m. and 2 p.m. and periods of minimum activity at 10 a.m. and 8 p.m. Both Oritz-Picon (1933) and Carleton (1934) described cycles of mitotic activity in the epidermis, but they found respectively that the maximum occurred during the day and during the night. Cooper and Franklin (1940) reported that the greatest epidermal mitotic activity in their mice was seen at about 10 a.m. and Blumenfeld (1942) found the maximum at 12 noon in mice and at 9 a.m. in rats.

In view of these somewhat conflicting results it was deemed advisable to establish the normal mitotic cycle in the strains of mice and rats intended for use in later experiments, in a strictly controlled external environment. It appears that environmental factors can markedly modify the mitotic cycle and this was confirmed by many observations of our own. The normal mitotic cycle having been established the effect of adrenaline and cortisone was investigated, and it was found that they depress mitotic activity.

The effect of adrenalectomy was then investigated, once it seemed possible that the removal of the source of these mitotic depressant hormones might lead to increased mitotic activity. The possibility of complicating factors due to operative procedure was borne in mind. They might lead to the liberation of adrenal hormones and thus affect mitotic activity in the early stages of the experiment. There was also the possibility of compensating mechanisms following adrenalectomy which might affect the final picture. Hence the time elapsing between operative procedure and examination of the mitotic cycle might be of importance. Therefore the mitotic activity was studied at 48 hr., and 18 days after operation.

The results suggested that a compensating mechanism did arise. To test this further the effect of exercise, a stimulus releasing adrenaline, on the mitotic activity of intact, sham-operated and adrenalectomized mice was determined.

MATERIALS AND METHODS

The animals used were WLL mice, CBA mice and Wistar rats aged 3–4 months. These were maintained in a strictly controlled external environment. Cages housing the animals were kept in a constant temperature chamber maintained at 20°. They were fed on a diet of rat cubes, oats, cooked flaked maize, dog biscuits and cod liver oil regularly between 9.45 and 10 a.m. each day. Natural and/or electric light was provided from approximately 8 a.m. to 6 p.m. Only after they were stabilized in this manner for three weeks or more were they used for experiments.

The mitosis rate was measured in the ear epidermis by means of the ear clip technique (Bullough, 1948*a*). Small pieces of the ear were removed at intervals, fixed in Bouin's alcoholic fluid, embedded in wax and cut into sections 7 μ thick. All stages of mitosis were counted in unit section lengths of 1 cm. of the ear epidermis, and from each ear clip 10 such counts were made and the average taken.

Adrenaline hydrochloride (Parke Davis and Co.) was used as a 1 : 1000 solution in water. *Cortisone acetate* in the aqueous vehicle No. 1, supplied by Merck and Co. was used.

RESULTS

Normal Mitotic Cycle in Mice and Rats

Ten male mice of both WLL and CBA strains were used in experiments 1 and 2, 10 rats of the Wistar strain were used in experiment 3. Fig. 1, 2 and 3 illustrate mitotic cycles seen in the 3 experiments.

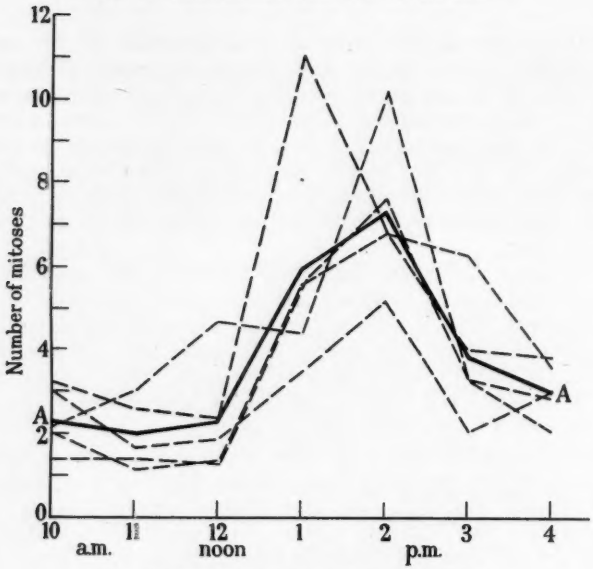

FIG. 1.—Normal mitotic cycle in WLL mice.
– – – – – – = Individual experiments.
A———— A = Mean.

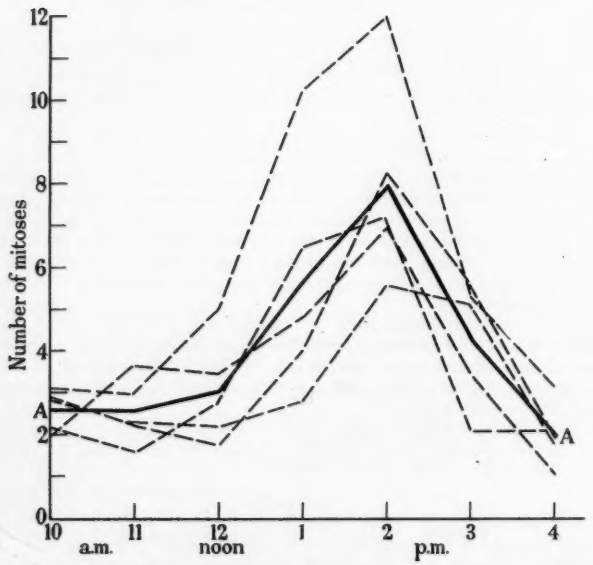

FIG. 2.—Normal mitotic cycle in CBA mice.
– – – – – – = Individual experiments.
A———— A = Mean.

In spite of considerable individual variation the mean pattern and magnitude of the rise and fall of mitotic activity is very similar in all 3 groups. Thus at the peak period (2 p.m.) there were 7·3 mitoses per cm. in WLL mice, 8 in CBA mice and 6·5 in the rats.

These findings confirm Bullough's (1948*a*) results with WLL and CBA mice and further show that the pattern of mitotic activity in Wistar rats is very similar to that in mice.

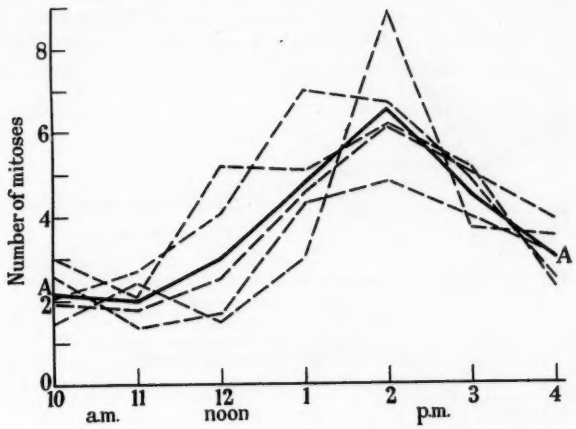

FIG. 3.—Normal mitotic cycle in Wistar rats.
– – – – – – = Individual experiments.
A———A = Mean.

Effect of Adrenaline on the Mitotic Cycle

Of 10 male mice of the WLL strain 5 received 0·04 mg./10g. mouse weight adrenaline subcutaneously, the injection being made immediately after the first ear clip at 10 a.m. Control animals received an equivalent volume (0·1 ml.) of 0·9 per cent saline.

The results (Fig. 4) show that adrenaline abolished the expected rise in the mitotic cycle. Thus at the peak period (2 p.m.) when the control animals showed a mean count of 6·8 mitoses per cm. the experimental animals showed only 0·2 mitoses and 2 hr. later the mean count had fallen to zero.

Effect of Cortisone

Of 10 male mice of the WLL strain 5 received cortisone, 2 mg./10g. mouse weight, subcutaneously, immediately after the first ear clip at 10 a.m. : 5 control animals received an equivalent volume of 0·9 per cent saline.

Results shown in Fig. 5 show that cortisone abolished the expected rise in the mitotic cycle. Thus at the peak period (2 p.m.) when the control animals showed a mean count of 7·3 mitoses per cm. the count in the experimental animals was 0·1 mitosis per cm. and 1 hr. later the mean count had fallen to zero.

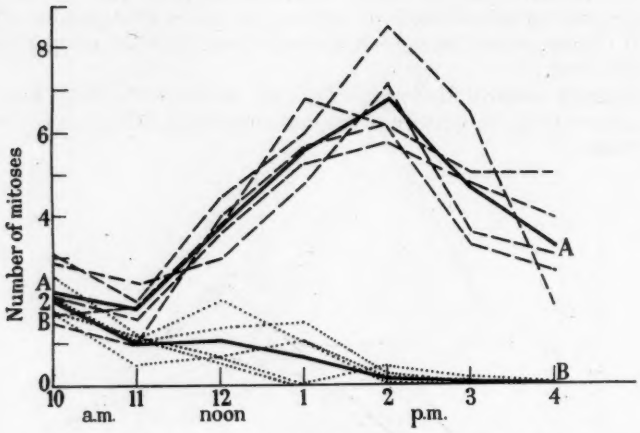

FIG. 4.—Effect of adrenaline on the mitotic cycle of WLL mice.
- - - - - - - = Individual controls.
A——A = Mean of controls.
. . . . . = Individual adrenaline-injected.
B——B = Mean of adrenaline-injected.

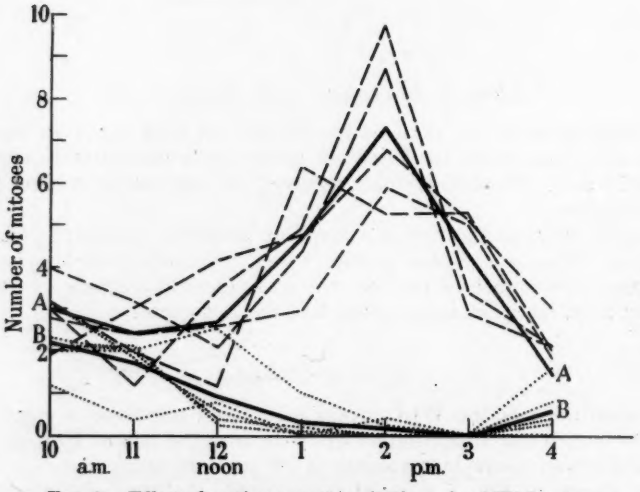

FIG. 5.—Effect of cortisone on the mitotic cycle of WLL mice.
- - - - - - - = Individual controls.
A——A = Mean of controls.
. . . . . = Individual cortisone-injected.
B——B = Mean of cortisone-injected.

Effect of Total Adrenalectomy and Sham Operation

Eight male CBA mice were used. In 4 mice both adrenal glands were removed under ether anaesthesia. After operation 1 per cent NaCl replaced ordinary drinking water. In 4 control animals a mock operation was performed. The technique was identical except that the adrenals were exposed to view but not removed. Forty-eight hours after operation ear clips were taken at hourly intervals from both groups, starting at 10 a.m. At the conclusion of the experiment the completeness of adrenalectomy was confirmed by killing the mice and examining the perinephric tissues macroscopically and microscopically.

Fig. 6 shows clearly that the mean pattern of mitotic activity differed from the normal in both control and experimental animals. In the controls mitotic activity

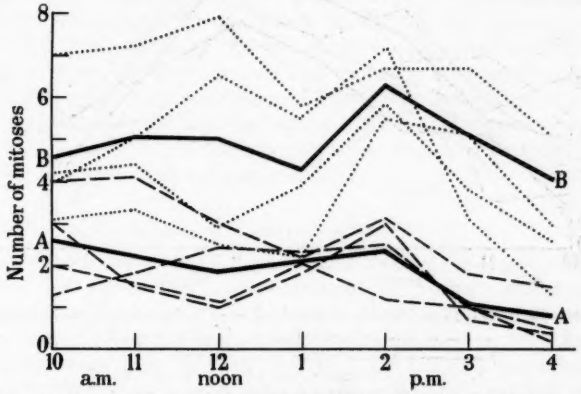

FIG. 6.—Effect of adrenalectomy on the mitotic cycle of CBA mice 48 hours after operation.
– – – – – – = Individual sham-operated.
A———A = Mean of sham-operated.
. . . . . = Individual adrenalectomized.
B———B = Mean of adrenalectomized.

was much depressed, this being particularly well demonstrated at the expected peak period (2 p.m.). In the adrenalectomized group, however, the peak of the curve is within normal limits although the rest of the points on the curve are somewhat elevated, suggesting that some stimulation of mitotic activity was occurring.

The next experiment was similar except that mitotic counts were made 18 days after operation. Fig. 7 shows that in both groups the pattern of mitotic activity is similar to that of normal animals.

Effect of Exercise in Normal and Adrenalectomized Rats

In this experiment there were 3 sham-operated, 6 adrenalectomized, and 3 intact control animals. Eighteen days after adrenalectomy, at 10 a.m., an ear clip was taken from all the animals in the various groups. All were then placed in a slowly revolving (80 rev. per hr.) cage driven by an electric motor. By this method the animals were prevented from going to sleep as they usually do during the course of an experiment, and were forced to change their position more or less

continuously. During the 6 hr. duration of the experiment it was estimated that the minimum distance which each rat had been forced to travel to maintain normal posture was approximately 480 yards (439 m.). Ear clips were taken at hourly intervals. Fig. 8 shows that there was a marked depression of mitotic activity in the intact and sham-operated controls while the adrenalectomized rats showed a fairly normal pattern.

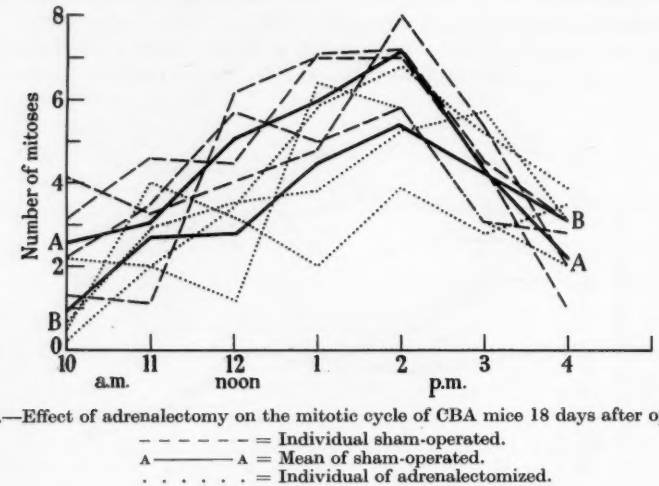

FIG. 7.—Effect of adrenalectomy on the mitotic cycle of CBA mice 18 days after operation.

– – – – – – = Individual sham-operated.
A ——— A = Mean of sham-operated.
. . . . . = Individual of adrenalectomized.
B ——— B = Mean of adrenalectomized.

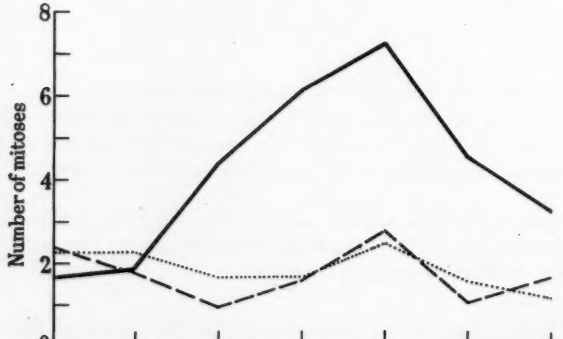

FIG. 8.—Effect of exercise on the mitotic cycle in adrenalectomized, sham-operated and normal Wistar rats.

——— = Adrenalectomized.
– – – – – – = Sham-operated.
. . . . . = Normal.

DISCUSSION

The normal mitotic cycle

The results show that mitotic activity in the epidermis of WLL mice, CBA mice, and Wistar rats is cyclical. Though there is a high degree of individual variation, maximum cell division is seen at approximately 2 p.m., and minimum at about 10 to 11 a.m. These results are similar to those of Bullough (1948*a*) for WLL and CBA mice, but also show that in Wistar rats the cycle is similar in magnitude and rhythm.

However, it must be stressed that this pattern of activity is seen only when the animals are maintained under strictly controlled environmental conditions. It would appear (Bullough, 1948 *a*, *b*) that the type of cycle common to any one colony is largely determined by the habits of resting and waking, and these will be determined by the routine of the laboratory. Personal experience bears out this hypothesis for it was found that if the morning feeding was delayed by one hour the peak of the mitotic curve occurred at 3 p.m. instead of 2 p.m. Further, there appears to be an inverse relation between bodily activity and mitotic activity (Bullough, 1948*b*) for a high mitotic activity is associated with sleep and rest and a low mitotic activity with waking and exercise.

Effect of adrenaline and cortisone on mitotic cycle

Green and Ghadially (1951) first demonstrated the powerful antimitotic activity shown by adrenaline and cortisone on mouse epidermis. In their preliminary report only counts at the peak period (2 p.m.) were made, but a prolonged depressant effect is clearly shown in the present work. Later, Bullough (1952*a*) obtained essentially similar results. He found that adrenaline had a powerful antimitotic action both *in vivo* and *in vitro* and that the same is true of cortisone. He also studied the effect of stress, induced by overcrowding adult male mice, on adrenal size and epidermal mitotic activity. After 3 weeks the size, expressed as the maximal sectional area, of the adrenal medulla of the crowded mice increased by about 80 per cent while that of the cortex increased by about 30 per cent. Simultaneously the epidermal mitotic rate fell by about 60 per cent. Bullough (1948*b*) has also shown that exercise depresses mitotic activity, and the results reported in this paper confirm this finding. This effect may largely be due to the release of adrenaline into the circulation.

In our cortisone experiments, control animals were injected with saline, as the aqueous suspending fluid was not available at that time. However, Bullough (1952*a*) found that this fluid had no mitotic depressant effect in the intact animal although it had such an effect in high concentrations *in vitro*. Hence the use of saline instead of the suspending agent does not affect our conclusions.

It appears that both adrenaline and cortisone exert their antimitotic effect in the antephase, *i.e.*, these compounds seem to have no effect on any mitoses already in progress. There is no piling-up of cells in one phase of mitosis such as is induced in metaphase by colchicine. The action of these hormones more resembles that of iodoacetate, cyanide and dinitrophenol which all inhibit mitosis in the antephase (Bullough and Johnson, 1951*a*, *b* ; Bullough, 1952*b*). Hence Bullough suggests that their mode of action may be similar, for all these substances interfere with some stage of carbohydrate metabolism, as do adrenaline and cortisone. The effects of cortical hormones on carbohydrate metabolism are not clear, but

they seem to stimulate gluconeogenesis and decrease carbohydrate utilization (Long, 1949). However, Laws (1952) and Roberts, Florey and Joklik (1952) do not support Bullough's hypothesis, for they fail to find a positive correlation between available glucose and mitotic activity.

Ebert and Barclay (1952) have shown that cortisone produces prolonged vascular spasm in the rabbit ear, and the vasoconstrictor effects of adrenaline have been known for a long time. It is therefore tempting to suggest that both adrenaline and cortisone produce their antimitotic effect by a diminution of blood supply and hence metabolites to the epidermal cells. However, the fact that these drugs can exert their action both *in vitro* and *in vivo* (Bullough, 1952*a*) seems to point against such a simple explanation. Nor could vasoconstriction explain the diminution of mitotic activity in the lymph nodes, suggested by the work of Baker *et al.* (1951) and in the liver by Roberts *et al.* (1952) for it seems unlikely that simultaneous vasoconstriction occurs in every part of the vascular tree as a result of cortisone. It is well known, of course, that adrenaline constricts the vessels in the skin, but not those in the muscles and splanchnic area.

Adrenaline stimulates the hypophysis to liberate ACTH, thereby inducing the corticoid secretion from the adrenal glands (Vogt, 1945; McDermott, Fry, Brobeck and Long, 1950; White, 1947). Hence at least part of the effect of adrenaline on mitotic activity may be an indirect one, mediated *viâ* the pituitary-adrenocortical system. That this is not the full answer is suggested by the fact that adrenaline can exert its effect both *in vitro* and *in vivo* (Bullough, 1952*a*).

The depression of mitotic activity first demonstrated by Green and Ghadially (1951) suggests a possible explanation for some of the known effects of cortisone. Thus several studies have indicated that the glucocorticoid hormones interfere with wound healing (Baker and Whitaker, 1950; Bangham, 1951; Billingham *et al.*, 1951). They caused depressed cellular proliferation, delayed healing, defective granulation tissue formation, etc. All this could be explained by a depressed mitotic activity in the various tissues in the region (Green, 1950). It has also been shown that ACTH suppresses the growth of epidermis, sebaceous glands and hair (Baker *et al.*, 1948; Baker and Whitaker, 1950) and that cortisone reduces the size of sebaceous glands (Castor and Baker, 1950). Such observations would accord with its antimitotic activity.

However, it is unlikely that cortisone depresses mitotic activity in every tissue in every species, and it would be unwise to generalize on the basis of a few observations. The work of Roberts *et al.* (1952) suggests that while cortisone depresses mitotic activity in the regenerating liver it does not do so in the intestinal epithelium of the mouse. Mitoses were also seen by these authors in lymphoid follicles in mice receiving cortisone, but they do not clearly state whether mitotic depression occurred in this tissue or not. Baker *et al.* (1951) have, however, suggested that mitotic depression does occur in lymphoid tissues. It must, however, be noted that the findings of Baker *et al.* (1951) and of Roberts *et al.* (1952) are based on subjective impressions about the number of mitoses seen in their preparations, and not on quantitative methods as have been employed by us and by Bullough.

Effect of adrenalectomy, sham-operation and exercise on mitotic cycle

The effect of adrenalectomy on the mitotic cycle was investigated, for it seemed possible that removal of the source of these mitotic depressant hormones might

lead to increased mitotic activity. The difficulty here was that both the anaesthetic and the operative trauma might themselves stimulate adrenal hormone output. This would only be a temporary effect, but also unknown compensating mechanisms might come into action for a time and tend to mask the true picture.

It was observed early that the pattern of mitotic activity was easily upset by any condition causing stress such as rough handling, chilling, struggling, etc. Stress-producing stimuli, such as over-crowding (Bullough, 1952*a*), and exercise (Bullough, 1948*b*), depress mitotic activity, and other examples are presented in this work.

With this point in mind, we can explain why the mitotic activity in the sham-operated group of animals is severely depressed 48 hr. after adrenalectomy while in adrenalectomized animals the peak of the curve is within normal limits and the rest of the curve is slightly higher than in normal mice. It appears that the stress caused by the sham-operation is responsible for the depressed mitotic activity. On the other hand, in the adrenalectomized group, mitosis continues and it can be concluded that the adrenal is the source of the mitotic depressant hormones. There is, in fact, evidence of some stimulation of mitotic activity due to the absence of the adrenals.

Eighteen days after operation both the adrenalectomized and sham-operated groups showed a more or less normal mitotic cycle. This is, of course, understandable in the sham-operated animals, but a heightened mitotic activity in the adrenalectomized group might be expected. We have no explanation of why this was not so, beyond the possibility of compensating mechanisms coming into play. That these adrenalectomized animals were not capable of normal hormone production is revealed by their response to exercise. Exercise depressed the mitotic activity of both intact controls and sham-operated animals, whereas in the adrenalectomized group it was not affected. These results strongly support the hypothesis that at least one of the mechanisms by which a stress-producing stimulus leads to depression of mitotic activity is by the liberation of adrenal hormones.

However, many systemic mitotic depressants act directly for they are active in tissue culture. Thus for instance, Green and Ghadially (1951) found that adenosine depresses epidermal mitotic activity not only in the intact but also in the adrenalectomized animal. Stoner and Green (1950) showed that adenosine did not stimulate adrenal cortical activity and Hughes (personal communication) has also observed in tissue culture experiments with chick-embryo fibroblasts that adenosine depresses mitotic activity. Nevertheless, the present work does establish that in whole animals, control of epidermal mitotic activity is intimately related to the adrenal gland.

SUMMARY

The mitotic cycle of epidermal cells has been studied in the ear of Wistar rats, CBA mice and WLL mice maintained under standard environmental conditions. Quantitatively and qualitatively the results obtained were very similar in all these animals. Mitotic activity was low at 10 a.m. and high at 2 p.m., under the conditions of the test.

Adrenaline and cortisone given by subcutaneous injection both severely depressed mitotic activity.

Some evidence of enhanced mitotic activity at other than the peak period was obtained in mice 48 hours after total adrenalectomy, but sham-operated controls showed a marked depression of mitotic activity.

Mitotic studies made on the ear epidermis 18 days after operation revealed a more or less normal mitotic cycle in both adrenalectomized and sham-operated animals.

Exercise caused no depression of mitotic activity in adrenalectomized animals but produced a powerful depression in controls with intact adrenals.

It is concluded that at least one of the mechanisms by which a stress-producing stimulus leads to a depression of mitotic activity is by the liberation of adrenal hormones.

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THE RELATIONSHIP OF TISSUE FORMATION AND DESTRUCTION TO THE SERUM PROTEIN-BOUND CARBOHYDRATE LEVELS IN EXPERIMENTAL INFLAMMATION

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DISAGREEMENT exists as to whether an elevation of serum total protein-bound carbohydrate depends on the formation or on the destruction of tissue. From a study of chronic inflammatory and malignant neoplastic states in man Seibert, Seibert, Atno and Campbell (1947) suggested that tissue destruction accounted for this rise, whereas Shetlar, Foster, Kelly, Shetlar, Bryan and Everett (1949) held the opposite view, namely that increased carbohydrate concentration could more reasonably be correlated with tissue proliferation. The serum carbohydrate levels have also been followed in dogs with experimental inflammatory lesions, the rise in concentration being correlated with tissue proliferation or repair (Shetlar, Bryan, Foster, Shetlar and Everett, 1949). In rats bearing the Walker 256 carcinoma Shetlar, Erwin and Everett (1950) related the increase in the serum protein-bound carbohydrate level to increasing size of the tumour, which Shetlar (1952) interpreted as a form of tissue proliferation.

The conclusions drawn from these investigations depend on correlation of the serum carbohydrate levels either with the clinical state of the patient or with the gross appearances of the experimentally induced conditions. Such procedures are unsatisfactory bases of assessment, since tissue formation and destruction commonly coexist in varying proportions both in inflammatory and in neoplastic states. Microscopical study is necessary to determine the exact types of change and their relative extent at different times. We have therefore reinvestigated the relationship of tissue formation and destruction to changes in the serum protein-bound carbohydrate levels in an experimentally induced inflammatory process. Particulate silica, given intraperitoneally, was adopted as the inflammatory stimulus on account of its well known fibrogenic capacity and because an acute reaction with necrosis is prominent in the early stages. Since cortisone suppresses fibrosis, *i.e.*, one kind of tissue formation, its use seemed a suitable means of aiding the isolation of the destructive element in the inflammatory process. The results indicate that elevation of serum protein-bound carbohydrate levels is connected with the acute phase of the inflammatory reaction and not with tissue formation as judged by fibrosis. A summary of these findings has already been published (Keyser and Heppleston, 1955).

METHODS

The rabbits were young adult males of mixed breed and weighing approximately 2500 g. Each animal received, by intraperitoneal injection without anaesthesia, 2 g. of Colin Stewart silica (particle size below 7 μ) suspended in 10 ml. physiological saline. Prior to administration the suspension was sterilized in an autoclave and then agitated in a Kahn shaker for

2 hr. Three-ml. samples of blood for biochemical analysis were collected from the ear at intervals. Repeated withdrawal of large amounts of blood was avoided as this might have caused haemodilution. All blood samples were taken after the rabbits had fasted for several hours. As a check on possible alterations in blood volume, blood for haemoglobin estimation (alkaline haematin method) was taken immediately before each blood sample collected for carbohydrate determination. In the week prior to starting the injections haemoglobin and carbohydrate determinations were performed on at least two occasions on every rabbit; each animal thus served as its own basis of interpretation.

Serial estimations of carbohydrate

Changes in the serum protein-bound carbohydrate levels were followed serially in 14 rabbits. In addition to the silica, 8 of these received cortisone acetate (Roussel) subcutaneously, 25 mg. being given on the day before the injection of silica, followed by 12·5 mg. daily thereafter. Most animals were killed after 5–6 weeks but a few (including animals receiving silica alone and with cortisone) were allowed to survive for several months. The general health remained good, although some rabbits on cortisone lost weight.

Terminal estimations of carbohydrate

To secure histological correlation a further experiment was necessary. Animals were killed at appropriate intervals, as indicated by the first experiment, and a single 20-ml. fasting sample of blood was taken from each rabbit just before killing. Twelve rabbits were used, six being given cortisone as before. At intervals up to 15 days animals were killed in pairs, one of each pair having had cortisone. There was no significant change in the weight of these rabbits.

Control experiments

To ensure that the observed variations in serum protein-bound carbohydrate concentration were not attributable simply to the administration of cortisone or saline, control experiments were carried out. Three rabbits received cortisone alone subcutaneously in similar dosage to previous experiments and three others 10 ml. sterile physiological saline by intraperitoneal injection. The carbohydrate and haemoglobin levels were followed by serial estimations for 2–3 weeks.

Histological procedures

Immediate autopsy was performed on all animals and the size and extent of the peritoneal lesions were noted as well as their character. Representative lesions from the gut, from the abdominal wall or diaphragm, and from the omentum or mesentery, together with any free-lying masses of dust, were fixed in neutral formol saline and embedded in paraffin. Sections were stained by haematoxylin-eosin and for reticulin fibres. Toluidine blue and alcian blue were used to study tissue polysaccharides and the effect of preliminary incubation for 3 hr. at 37° with hyaluronidase ("Hyalase", Benger) on staining reactions with both methods was determined. The periodic acid-Schiff reaction was also applied. The Feulgen reaction was employed to demonstrate the remains of nuclear material.

These histological observations were supplemented by others obtained from preliminary experiments on 6 rabbits in which 200 mg. quartz (particle size below 5 μ) in suspension was given intraperitoneally together with cortisone subcutaneously in some of them. Though producing characteristic lesions this amount of silica did not provide an adequate rise in serum protein-bound carbohydrate concentration.

Biochemical procedures

The chemical procedures employed have been described previously (Keyser, 1954), serum total protein being separated by alcoholic precipitation, albumin with 26 per cent Na_2SO_4, and mucoprotein by the method of Winzler, Devor, Mehl and Smyth (1948). The carbohydrate was estimated by the orcinol method essentially as described by Rimington (1940).

It is known that filtrates obtained by fractionation of serum with 26 per cent Na_2SO_4 may contain small amounts of both α- and β-globulins (Jager, Schwartz, Smith, Nickerson

and Brown, 1950). On this account and also to distinguish the carbohydrate components of the different serum proteins, an attempt was made to apply the quantitative method of Köiw and Grönwall (1952). However, in the rabbit the carbohydrate bands corresponding to the globulin fractions were not sufficiently well separated to permit accurate quantitative measurements, as was also noted by Sohar, Bossak and Adlersberg (1955). Furthermore, the amount of albumin carbohydrate was so small that minor variations could not be detected. This method was therefore abandoned.

RESULTS

Serum Protein-bound Carbohydrate

The normal initial values found are given in Table I.

TABLE I.—*Normal Serum Protein-bound Carbohydrate Values*

	Number of rabbits	Number of estimations	mg. hexose/100 ml. (mean)	S.D.
Total protein-bound carbohydrate	33	71	92	10
Albumin-bound carbohydrate	32	68	12	2·5
Mucoprotein-bound carbohydrate	8	19	10	2·5

Since the object was to determine the relationship between the histological features of the inflammatory process and the serum protein-bound carbohydrate levels, it is considered sufficient to present the changes in these levels graphically. To compare the experimental and control groups, all carbohydrate determinations in each rabbit have been expressed as a percentage of the average normal value for that animal. Values from more than one rabbit were usually obtained at the same period of the experiment and the average was entered in the graphs. As the same trend was evident in experiments involving both serial and terminal estimations of carbohydrate, the results were combined in the graphs. It was impracticable to perform blood volume estimations but adjustment of the carbohydrate levels in proportion to variations in the haemoglobin concentration (the base-line being the average normal concentration measured before the experiment) did not affect the shape of the graphs. It is therefore unlikely that the results were complicated by haemodilution or haemoconcentration. Calculation of the total protein- and albumin-bound carbohydrate as a percentage of total protein was essentially without effect on the shape of these graphs.

Total Protein-bound Carbohydrate

After the injection of silica there was a sharp rise in the total protein-bound carbohydrate (T.P.C.), which reached a maximum in 1–3 days (Fig. 1). This rise was followed by a slower fall, the concentration of carbohydrate approaching the base-line level by about the 10th day. In animals receiving silica alone the carbohydrate content of the serum total protein rose from a mean of 1·47 per cent, to a peak of 2·29 per cent on the second day. In animals receiving silica and cortisone the corresponding values were 1·57 per cent and 2·15 per cent, the peak value again occurring on the second day. Cortisone, therefore, did not prevent the rise in T.P.C. induced by silica. Control injection of saline intraperitoneally had little influence on the carbohydrate levels. The slight elevation of

T.P.C. on the first and second day after cortisone alone rapidly subsided. The high value after 43 days on cortisone may be unrelated to the drug, since this reading is the average of estimations on only two rabbits. One of these gave a value that was little elevated, while the other rabbit had a particularly high value and showed liver necrosis and a fungus infection when killed at 123 days.

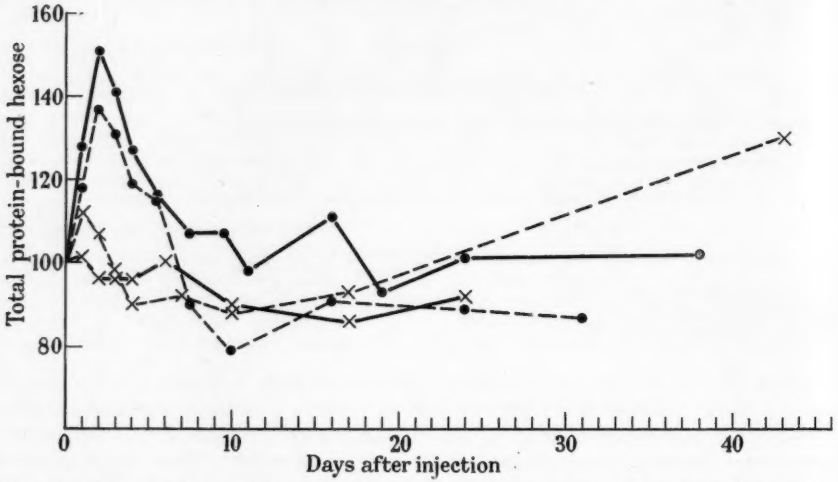


FIG. 1.—Serum total protein-bound carbohydrate (hexose), expressed as a percentage of base-line value, after silica alone (●————●), silica + cortisone (●- - - -)●, saline alone (×————×), and cortisone alone (× - - - - ×).

Albumin-bound Carbohydrate

The curves for albumin-bound carbohydrate (A.C.) resemble those obtained for T.P.C., but the fall of the levels in the silica + cortisone group was more protracted (Fig. 2). We are unable to account for the raised values found on the 10th and 17th days after cortisone alone. Expressed as a percentage of serum total protein, the albumin-bound hexose rose from a mean of 0·20 per cent to a peak of 0·40 per cent on the second day after the injection in animals receiving silica alone. In the silica + cortisone group the corresponding figures were 0·20 and 0·41 per cent, the peak value again being found on the second day. In one animal from each group, serial determinations of albumin nitrogen were made and the levels remained almost constant although the A.C. showed the usual rise.

Mucoprotein-bound Carbohydrate

In rabbits on which terminal carbohydrate estimations were made, large samples of blood were available and permitted the estimation of absolute mucoprotein-bound carbohydrate (M.C.) levels in addition to T.P.C. and A.C. (Table II). As far as can be judged from single observations on different animals at different times and over a short period, the peak level of M.C. occurred at about the same time as with the T.P.C. and A.C. levels.

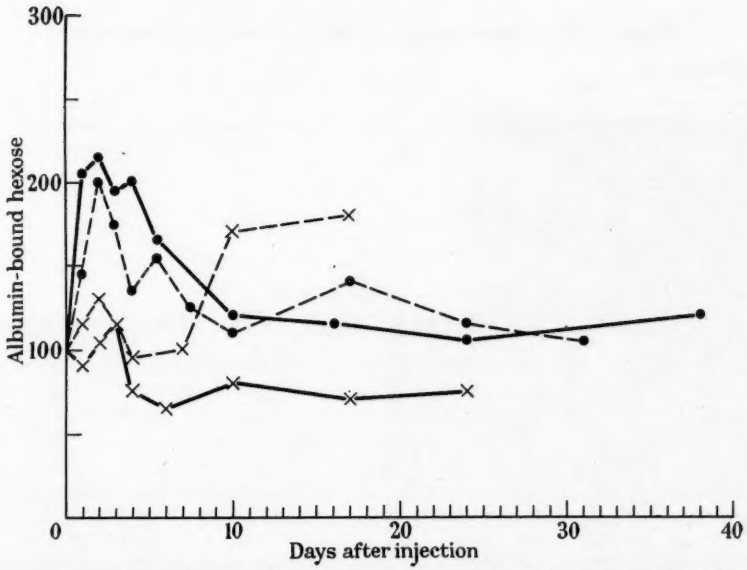


FIG. 2.—Serum albumin-bound carbohydrate (hexose), expressed as a percentage of baseline value, after silica alone (●————●), silica + cortisone (● - - - - ●), saline alone (×————×), and cortisone alone (× - - - - ×).

TABLE II.—*Serum Mucoprotein-bound Carbohydrate*

Days	mg. hexose/100 ml. After silica alone	After silica + cortisone
1	16·5	10
2	16	25
3	15	14·5
5	10·5	13
8	15	17
15	10	13·5

For normal range of values see Table I. Each value is from a different rabbit.

Histological Correlation

The inflammatory stimulus applied to the rabbits was uniform, since each was injected with the same amount of silica. Furthermore, the distribution and size of the resulting lesions in the first few days of the experiment were similar in animals whether or not they received cortisone. Later in the experiment the size of the lesions was influenced by differences in the degree of cellular proliferation and fibrosis in the two groups.

A quantitative assessment of the main characteristics of the inflammatory response at different stages is given in Table III. The various features are not always present to the same degree in lesions from different sites in the same animal. Lesions from the omentum and mesentery tended to show the more severe changes and free-lying dust masses (cortisone-treated rabbits) the least

TABLE III.—*Characteristics of the Inflammatory Response*

Experiment				Dust			
Duration in days	Type	Polymorph exudation	Cellular necrosis	Free	In macrophages	Fibroblasts	Fibres
2–3	S	+++	+++	++++	Minimal	+++	++
	S and C	+++	+++	++++	,,	+	+
7–8	S	++	++	+++	+	++++	++++
	S and C	+ to ++	++	++++	Minimal	+ to ++	+ to ++
3–5 weeks	S	+	+	Minimal to +	++++ to +++	+++	+++++
	S and C	+	+	+++	+	+	++

S = Silica intraperitoneally.
C = Cortisone subcutaneously.

severe. In view of this the features of all lesions were graded on three separate occasions without reference to each other and good agreement was reached. Account has been taken of these differences in assessing the response of individual animals and an overall grading was then made for each experimental group. Polymorph exudation implies the presence of well preserved cells. Cellular necrosis, judged largely by the Feulgen reaction for nuclear deoxyribonucleic acid, mainly concerns polymorphs, but macrophages almost certainly comprise a proportion of the necrotic cells. In the lesions from some rabbits, reticulin fibres exist in masses of dust devoid of demonstrable cellular elements. Since cells are apparently concerned in the formation of reticulin it seems that fibroblasts too share in the cellular necrosis. The characteristics of representative lesions are illustrated in Fig. 3–6.

Wherever fibroblasts were actively proliferating the ground substance in the lesions showed toluidine blue metachromasia and stained with alcian blue, both reactions being totally or largely abolished by previous incubation with hyaluronidase. The ground substance did not give a positive periodic acid-Schiff reaction. These findings indicate the presence in the ground substance of a mucopolysaccharide of acidic nature (Pearse, 1953 ; Braden, 1955).

Two to three days after the injection of silica without cortisone, the inflammatory reaction includes the presence of numerous polymorphs, many being well preserved though many are necrotic, but there is little dust phagocytosis. Fibroblastic proliferation is well developed, reticulin fibres are being formed and acid mucopolysaccharide occurs in the associated ground substance. The rapid rise in serum T.P.C. and A.C. closely paralleled this early acute inflammation.

EXPLANATION OF PLATE

FIG. 3.—Silica alone. Margin of a 2-day-old lesion on the peritoneal surface of the gut. Numerous polymorphs around and in the mass of dust (seen above). Haematoxylin-eosin. × 130. Similar changes were seen in the silica + cortisone group at the corresponding period.

FIG. 4.—Silica alone. Centre of a 3-day-old lesion on the gut showing numerous necrotic polymorphs in the dust. H. & E. × 130. Similar changes were seen in the silica + cortisone group at the corresponding period.

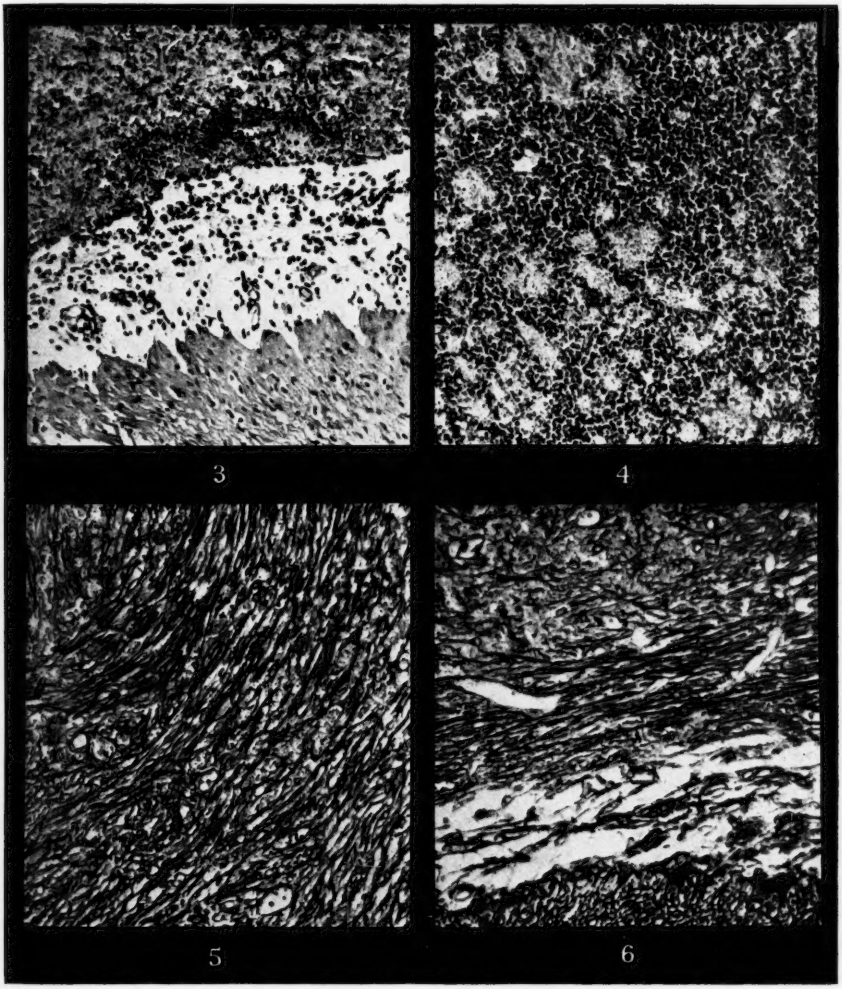
FIG. 5.—Silica alone. Approximately a quarter of an 8-day-old lesion from the gut showing dense reticulin fibres between dust phagocytes. × 130.

FIG. 6.—Silica + cortisone. 8-day-old lesion from the gut (seen below) showing the full extent of the reticulin fibrosis. × 130.

ERRATUM.

Vol. XXXVIII, No. 1, February 1957, p. 116 (A. G. Heppleston and J. W. Keyser).

In Table III the last entry in the left-hand column, "3–5", should be "3–5 weeks".



3 4

5 6

At 7–8 days the silica lesions show increased fibroblastic activity and fibre formation, while dust phagocytosis is now more conspicuous. The T.P.C. and A.C. levels, however, are falling and at the same time polymorph exudation and cellular necrosis are less evident, as is mucopolysaccharide in the ground substance. Several weeks after injection of silica, phagocytosis is greatly increased and fibrosis, though not now so active, is more extensive and more collagenous. Acute inflammation and necrosis are still further diminished, mucopolysaccharide in the ground substance is in general scanty and the carbohydrate levels remain approximately normal.

Cortisone is without appreciable effect on polymorph exudation and cellular necrosis, but at all stages it diminishes considerably the proliferation of fibroblasts and fibre formation as well as macrophagocytosis. Mucopolysaccharide in the ground substance is reduced in proportion to the depression of fibroblastic activity. It must be added that each of the rabbits receiving cortisone showed the usual enlargement and increased glycogen content of the liver (Constantinides, Szasz and Durrach, 1955; Businco, 1954; Schmid-Bircher, 1954; Williams and Lowe, 1954). Cortisone prevents neither the early rise nor the subsequent fall in the levels of T.P.C., A.C. or M.C. Although the graphs might suggest that cortisone slightly inhibits the rise in T.P.C. and A.C. following the injection of silica, there was in fact considerable overlap between the silica and silica + cortisone groups. The difference does not therefore appear to be significant, but an experiment involving many more animals would be needed to elucidate this point statistically.

An incidental finding in rabbits receiving silica and cortisone for over 30 days was a ragged gelatinous exudate on the peritoneal surface of the abdominal wall and adjacent gut, resembling that observed by Magarey and Gough (1952). This exudate was widespread and not confined to the vicinity of silica aggregates. Histologically, it consisted of thin-walled blood vessels with scattered histiocytes and fibroblasts lying in ground substance, which in most animals stained patchily with alcian blue. This reaction was abolished by prior incubation with hyaluronidase, indicating the presence of acid mucopolysaccharide. The non-reacting areas in the ground substance gave the impression of spaces from which material had been dissolved. At the base of the gelatinous exudate in rabbits given cortisone for over 90 days fibroblastic proliferation and fibre formation occurred and were associated with toluidine blue metachromasia (abolished by hyaluronidase) in the ground substance. The most abundant gelatinous exudate was seen in a control rabbit on cortisone alone for 123 days. This animal also had liver necrosis and a fungus infection (probably aspergillosis) of the pleura and large gut. A feature common to all the rabbits showing the exudate was sarcosporidiosis affecting the muscle fibres of the abdominal wall. In the present experiments none of the rabbits receiving only silica developed the gelatinous exudate and none showed sarcosporidia. To establish an association between cortisone and the parasite in the production of this peculiar exudate, more observations are needed on the effect of cortisone on rabbits with and without sarcosporidiosis.

DISCUSSION

The results suggest that the rise in serum T.P.C. and A.C. levels is related to the early acute inflammation induced by silica. The rise cannot be correlated

with tissue formation as judged by the fibroblastic and fibrogenic responses, nor with macrophagocytosis, since these elements become prominent when carbohydrate levels are falling. The selective modifications effected in the inflammatory response by cortisone support these conclusions. The acute inflammatory reaction to silica still occurs when the serum carbohydrate concentration increases and the fall follows the same general course despite partial inhibition of the productive components of the inflammatory process. The parts played by local factors, such as cellular necrosis and depolymerization of ground substance, in the elevation of serum carbohydrate remain to be determined. The amount of mucopolysaccharide demonstrated in the ground substance of the lesions is related to fibrogenesis but not apparently to the concentration of serum protein-bound carbohydrate. Attention might also be directed to the functional activity of the liver, since it normally contributes much, if not all, of the glycoproteins and mucoproteins in the serum (Werner, 1949 ; Miller and Bale, 1954 ; Miller, 1955).

The present findings therefore do not support the conclusion of Shetlar, Bryan *et al.* (1949) that in experimental inflammation tissue formation is responsible for the rise in serum total protein-bound carbohydrate. Our results are also opposed to Shetlar's (1952) view that the rise in concentration of serum albumin-bound carbohydrate depends on tissue proliferation. We are not yet in a position to assess the part played by tissue destruction. It may be mentioned that, in patients with various forms of muscular atrophy and dystrophy, not only may the T.P.C. and M.C. levels be increased but also the level of A.C. (Keyser, Jenkins, Lloyd, Pallis and Sanders, unpublished). In such diseases muscle wasting is associated with fat replacement but not with fibroblastic proliferation, and such increase of fibrous tissue as occurs may well represent condensation of pre-existing connective tissue rather than new formation.

SUMMARY

Elevation of the serum protein-bound (total and albumin) carbohydrate closely paralleled the early acute inflammation induced in the peritoneal cavity of rabbits by silica and the levels fell when silicotic fibrosis and macrophage response became prominent.

Cortisone did not materially affect the rise or fall in concentration of carbohydrate, and the acute inflammatory reaction did not appear to be significantly modified ; fibrosis and macrophagocytosis were, however, considerably inhibited.

In these experiments, therefore, increase in serum protein-bound carbohydrate concentration can be correlated with acute inflammation but not with tissue formation as judged by fibrosis or macrophage response.

The factors responsible for the rise in carbohydrate levels remain to be determined.

We are indebted to the Medical Research Council for supplying the cortisone, and to Mr. J. P. Napper for photographic assistance.

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" PSEUDO-LYMPHOCYTIC CHORIOMENINGITIS ": A CORRECTION

F. O. MacCALLUM, T. F. M. SCOTT, G. DALLDORF AND REBECCA GIFFORD

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MacCallum, Findlay and Scott (1939) reported the isolation of two strains of a virus which they called pseudo-lymphocytic choriomeningitis (P.LCM), from the brains of mice inoculated intracerebrally with cerebrospinal fluid from two patients with non-bacterial meningitis occurring 8 months apart. The records of the original experiments are no longer available but it is known that the mice used were bought from a dealer and not bred in the laboratory. No experiments on ectromelia were carried out in the laboratory, and the disease was not recognized in the large numbers of mice used at the time for different virus investigations.

The viruses described were isolated by brain to brain passage in mice, and by the 3rd passage in each case the incubation period to apparent onset of illness was 4–5 days, and the mice showed the type of spasm on spinning by the tail considered at the time to be characteristic of the mouse virus known as lymphocytic choriomeningitis (LCM) in which the incubation period was, however, never less than 6 days and usually 7–8 days following intracerebral inoculation. The new viruses were related to each other but not identical. There was no apparent immunological relationship between the two new viruses and LCM.

One patient appeared to develop a low level of neutralizing antibody in convalescence but the other, tested only at 1 year, was negative. Attempts to produce immune serum by intraperitoneal inoculation of rabbits were unsuccessful.

Histological examination of the brains of mice with P.LCM often showed little pathological change. At other times the picture was similar to that seen in LCM but round cell infiltration was not as extensive. No changes were detectable in any other organ.

In their paper MacCallum *et al.* stated that the " virus is readily maintained on the chorioallantoic membrane of the developing chick embryo but has not been grown successfully in culture of chick or mouse embryo tissue in serum-Tyrode medium ".

In the period 1939–45 the virus was passed from time to time in mice but records of this are no longer available. The oldest sample of virus now available is one dried in January 1946 and of an unstated number of mouse passages.

Little further work was carried out on this virus by any of the original authors except for occasional unsuccessful attempts to find antibodies to it in cases of aseptic meningitis.

One of us (G. D.), who has been particularly interested in various unusual human and mouse viruses, identified two apparently new neurotropic agents, supposedly of human origin, as ectromelia and observed that ectromelia can mimic LCM in mice inoculated intracerebrally (Dalldorf and Gifford, 1955). The two strains, which had been sent from Europe for study, both induced the following changes in experimental mice : cyanosis and a peculiar plum colour, followed by sudden death in suckling mice inoculated intracerebrally or intraperitoneally ; tremors and convulsions in weaned mice, indistinguishable from those induced by LCM, except that they occurred on the 4th or 5th day instead of on the 6th or 7th day following intracerebral injection ; severe swelling and scab formation in the feet of weaned mice inoculated in the foot pads ; marked yellowness of the livers and haemorrhages of the spleens, particularly in mice injected intraperitoneally ; and the formation of inclusion bodies in the viscera and skin of affected animals. Because the original description of P.LCM stated that convulsions like those of LCM occurred early after inoculation of mice, the suspicion arose that P.LCM might be ectromelia. Consequently, a lyophilized sample of the virus, obtained from England in May of 1955, was injected in Albany mice by several routes. The results proved the suspicion to have been well founded, for the mice responded exactly like those infected with the two " unknown " strains from Europe.

This was reported to F. O. M. who inoculated several ampoules of the virus dried in 1946 on to the chorioallantoic membrane (CAM) of 10–12-day-old fertile hen eggs. Typical lesions of ectromelia appeared after 48–72 hr. incubation at 36°. The appearance of the lesions could be prevented by prior incubation of the inoculum with ectromelia immune serum from mice and vaccinia immune serum from a rabbit. Attempts to recover any other agent in the brains of mice of various ages by intracerebral inoculation of mixtures of the virus and immune vaccinia or ectromelia sera have been unsuccessful. T. M. S. then found the strain of P.LCM in his possession gave the same results. The lesions on the CAM of hen eggs are so obvious that it seems doubtful that if present in the original experiments they were not seen. They are not mentioned in the original paper and F. O. M. and T. F. M. S. have no recollection of seeing them but that was 17 years ago!

CONCLUSION

The virus of pseudo-lymphocytic choriomeningitis no longer exists as such. Whether it was originally a definite entity or only ectromelia virus will never be known.

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